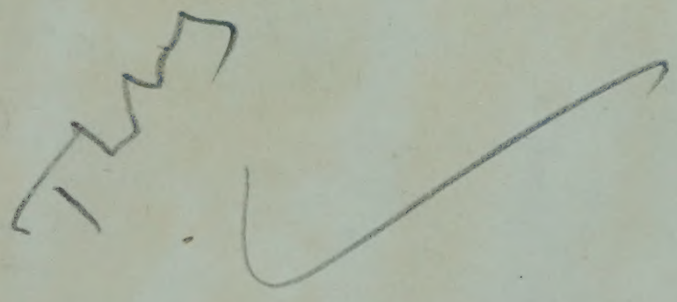
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20 " mycology
21 " parasitology. P.T.O.



A TEXTBOOK OF CLINICAL PATHOLOGY

Edited by

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Washington, D. C.



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PREFACE

The *Textbook of Clinical Pathology* is designed to give the medical student, interne, resident physician, clinical pathologist, and teacher of medicine an authoritative source of information on how to use the clinical laboratory most advantageously. It portrays what tests to order, when in the course of disease to order them, how to interpret and evaluate the results; in short, how best to use the clinical laboratory in differential diagnosis and in following the course of disease in patients. This approach is coupled with continued emphasis on normal function and the mechanism of disease. It is the hope of the authors that this text will fill a real need in assisting the physician to use the clinical laboratory to greater advantage and to gain a better understanding of how to interpret and evaluate clinical laboratory reports.

Continuing the major reorganization of material and drastic redesign of chapters started in the fourth edition, completely new chapters on medical bacteriology, medical mycology, and medical parasitology have been added. These chapters cover succinctly types of infections which frequently are poorly understood, with resulting poor diagnosis.

The chapters on renal function tests and urine examination have been largely redesigned and combined, permitting a better presentation of kidney function and physiology, and establishing a better basis for understanding kidney pathology and results of the various tests.

Two additional new chapters have been developed from portions of previous material—Chapter 24, Seminal Fluid, and Chapter 25, Feces. In addition, the chapter on sputum has been redesigned and expanded, incorporating new material on saliva and bronchial aspirates.

Rapid advances and many changes in the field of hematology have necessitated extensive changes in the first six chapters, particularly in the field of immunohematology.

Likewise, the chapter on liver function tests has been completely redesigned, emphasizing practical procedures in a diagnostic workup and the significance of the laboratory results.

The chapter on immunologic tests also has been extensively rewritten, emphasizing the mechanism and diagnostic values of various immunologic tests.

The chapter on the assay of vitamins and the chapter on the assay of hormones have been extensively rewritten, incorporating the numerous recent advances in these fields.

The chapters on syphilis serology and cerebrospinal fluid have been

largely rewritten, incorporating new materials and emphasizing the value of various diagnostic procedures.

The value of cytologic examinations in the early diagnosis of cancer has been appropriately emphasized by new material in several chapters dealing with the examination of body fluids, vaginal secretions, cervical smears, sputum, bronchial aspirates, and urine.

I wish to express my sincere gratitude to all the contributors for their long, painstaking, untiring efforts, often at considerable personal sacrifice, in the production of this fifth edition of the *Textbook of Clinical Pathology*. Also, I wish to express my gratitude to Miss Dorothy Sturm, of Memphis, who produced many of the illustrations in the hematology section; to Dr. Paul Weinstein for his painstaking review of the chapter on Medical Parasitology; and to Drs. John Cutler and Harold Magnuson for their constructive suggestions and assistance with the chapter on Syphilis Serology.

The index in the preceding edition has been roundly criticized for its brevity and inadequacies. In this edition much attention and vast amounts of time and energy have been devoted to the production of a very detailed index with ample cross-indexing. It is thus hoped to make the text more useful to the student.

S. E. M.

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CHAPTER 1

BLOOD TECHNIQUES

By L. W. Diggs

BLOOD COLLECTION

The proper collection of body fluids is essential for satisfactory examination. Since blood examinations occupy such a prominent place in laboratory procedures, and since so much of the practice of medicine revolves around the point of a needle, it is important to consider in detail various needle techniques and blood collection procedures.

Blood should be collected in chemically clean or sterile containers, for blood is an excellent culture medium for certain types of bacteria and fungi. Containers should be thoroughly rinsed and dried after washing. Soaps, water and cleaning solutions may alter the cells or introduce error in chemical tests. Blood specimens to be sent through the mails should be collected under sterile conditions, placed in tightly stoppered non-collapsible containers and packed with sufficient absorbent material so that none of the fluid will run out of the container if the specimen bottle is broken or the stopper becomes loose. The specimen should be sent by the fastest and most direct route.

It is best to collect blood in the morning before the patient has eaten, because the patient is under basal conditions and interfering food substances are least present at this time. Also, the early collection of blood specimens enables the laboratory staff to plan the day's work, to examine specimens promptly and to get out reports.

All specimens should be labelled carefully at the time the blood is taken, giving the name of the patient, date and other pertinent information. Gummed labels or glass-marking pencils are most commonly used. Laboratory request slips giving necessary information and stating the exact type of examinations desired should accompany the blood specimen. In making reports, the fluid examined, method of collection, the results, date and examiner must be clearly written and duplicate reports kept in the laboratory so that reference can be made if the report sent to the doctor is lost.

The cytological and chemical composition of the blood removed by venipuncture and by skin puncture are essentially the same.

In the majority of routine hematological procedures, whole blood is processed before it clots. In other procedures, the blood is allowed to

clot and the examinations are made on the serum expressed from the clot. Slanting of the tube before clotting makes it easier to collect the serum. The yield of serum is favored by loosening the clot with an applicator and centrifuging. Serum is used for the majority of precipitin and agglutination tests, for quantitative pigment estimations and for numerous chemical examinations. Clotted blood is preferred for the preservation of cells for typing, cross matching and Rh factor determinations, because the cells keep better in their own serum than in so-called isotonic salt solutions.

Blood may be defibrinated by agitation with an applicator or glass beads during the process of clotting. The fibrin strands stick to the foreign body and are removed. Defibrinated blood may be used for morphological studies and for various serological tests. Because some of the red cells are destroyed by mechanical agitation, defibrinated blood cannot be used for cell counts or pigment studies.

For many examinations, anticoagulants, such as oxalates, citrates, fluorides and heparin, are used. The blood may be examined whole or the cells separated by centrifugation or settling and the supernatant plasma used. The majority of routine hematological procedures can be done on oxalated blood, but care must be taken that the proper amount of anticoagulant is used and that the blood is shaken well both immediately after being drawn and before the specimen is examined. The examination must be made before deterioration occurs. If examinations cannot be made immediately, the specimen should be refrigerated.

Anticoagulants

Oxalates. Oxalates prevent the coagulation of blood by uniting with the calcium to form insoluble calcium oxalate. The following oxalate preparations are used:

1. Dried potassium oxalate. The final dilution recommended is 2 mg. per ml. of blood. For 10 ml. of blood, place 1 ml. of a 2 per cent aqueous solution in the container and dry in a warming oven at a temperature not exceeding 80°C. Overheating causes conversion of the oxalate into carbonate. Since dried potassium oxalate causes distortion and shrinkage of cells, it is not recommended for differential counts or hematocrit determinations.

Dried sodium or lithium oxalate can be substituted for potassium oxalate, using 2 mg. of the dried salt per 1 ml. of blood. Lithium oxalate has the advantage that it does not introduce an element which is tested for in normal blood.

2. Dried ammonium and potassium oxalate (Heller and Paul). A stock solution containing 1.2 gm. of ammonium oxalate and 0.8 gm. of potassium oxalate per 100 ml. of water is prepared. One ml. of this mixture, placed in a container and allowed to dry, is sufficient to prevent the coagulation of 10 ml. of blood. The mixture of ammonium and potassium oxalate has the advantage over potassium oxalate in that it does not produce significant shrinkage or enlargement of the red cells. It is preferred for sedimentation rate and cell volume determinations. Ammonium oxalate cannot be used for urea and nitrogen determinations.

3. Saturated potassium oxalate. Twenty grams of dried potassium oxalate are added to 100 ml. of distilled water and mixed. The undissolved salt is allowed to settle. One drop of the supernatant fluid is sufficient to prevent coagulation of 10 ml. of blood. A saturated solution of potassium oxalate mixes more readily with the blood than does the dried powder and this is particularly important when blood is collected in small test tubes. Another advantage in using saturated potassium oxalate is that the amount can be varied at the time of collection to suit the amount of blood needed. Disadvantages of the procedure are that the cells are distorted and a slight error in dilution is caused. One drop of fluid in 10 ml. introduces such a small dilution error that fluids so prepared can be used for cell counts, hemoglobin determinations and chemical tests requiring whole blood or plasma.

4. Isotonic oxalate. There are differences of opinion as to the ideal concentration of sodium or potassium oxalate which will prevent coagulation and cause minimal cellular distortion, change in cell size and hemolysis. It is generally agreed that the optimal concentration lies between 1.1 and 1.6 per cent. Concentrations below 1.0 per cent will cause hemolysis and those above 2 per cent interfere with various procedures. The use of 1 ml. of 1.4 per cent aqueous sodium oxalate for 5 ml. of blood has been widely used and is recommended as the concentration of choice for hematocrit determinations. With this concentration and this amount there is no hemolysis and the smears and reticulocyte preparations from such oxalate-blood mixtures are comparable to those made with fresh whole blood. The supernatant plasma from this mixture can be used for prothrombin determinations.

Fluorides. Potassium fluoride forms a weakly dissociated calcium component and thus prevents coagulation. Potassium fluoride interferes with the enzyme system having to do with glycolysis and is used for specimens collected for glucose determinations. The simplest preparation is 1 drop of a 40 per cent aqueous solution of potassium fluoride

for 5 ml. of blood. Sodium fluoride can also be used. It may be used in conjunction with oxalate as follows:

Potassium oxalate	10 gm.
Sodium fluoride	10 gm.
Distilled water	100 ml.

Mix. Shake well. Filter.

For 10 ml. of blood, measure out 0.5 ml. into tube or bottle. Dry by gently heating. Add beads, stopper and label.

Twenty parts of sodium fluoride and one part of thymol may be mixed and 10 mg. of the mixture placed in the container to preserve and to prevent clotting of 10 ml. of blood. A small spoon is useful for measuring.

Sodium Citrate. Sodium citrate forms a double salt with calcium. It is widely used for collecting blood to be used for transfusion since it is a relatively non-toxic salt which is rapidly utilized by the body or excreted by the kidneys. There is no agreement concerning the concentration of sodium citrate to be used for transfusion, but a commonly used preparation is 100 ml. of a 2.5 per cent solution for 500 ml. of blood. The citrate solution is sterilized in the collection flask and the blood from the donor allowed to run in. The addition of glucose and citric acid to the sodium citrate gives better preservative qualities. Sodium citrate is used in the Cutler sedimentation test (0.5 ml. of a 3 per cent aqueous solution and 4.5 ml. of blood). For the collection of sheep cells for the Wassermann reaction 30 ml. of a 10 per cent solution of sodium citrate in physiological saline and 2 ml. of formalin are used for each liter of blood.

Heparin. 0.1 ml. of liquid heparin or 1 mg. of dry heparin can be used for 10 ml. of blood. Heparin is useful for hematocrit studies, sedimentation rates, fragility tests and for collection of blood, for it does not alter the size of cells and is unlikely to cause hemolysis. It is not suitable for the Wassermann reaction or for agglutination tests.

Technic of Collection

Skin Puncture

The instruments of choice for skin puncture are the "Hemolet" disposable, venipuncture, and Hagedorn needles and the Bard-Parker blade (No. 11).

Each lancet must be heat sterilized before use to avoid the transmission of virus hepatitis. Venipuncture needles are placed in a small section

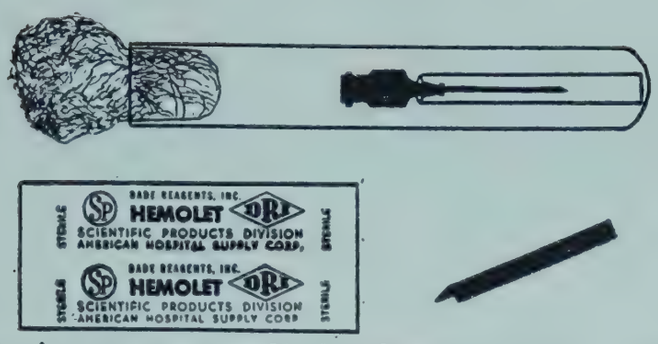


FIG. 1.1. Top: Venipuncture needle in glass cylinder. Steam sterilized in test tube with cotton plug. Bottom: Disposable "Hemolet."

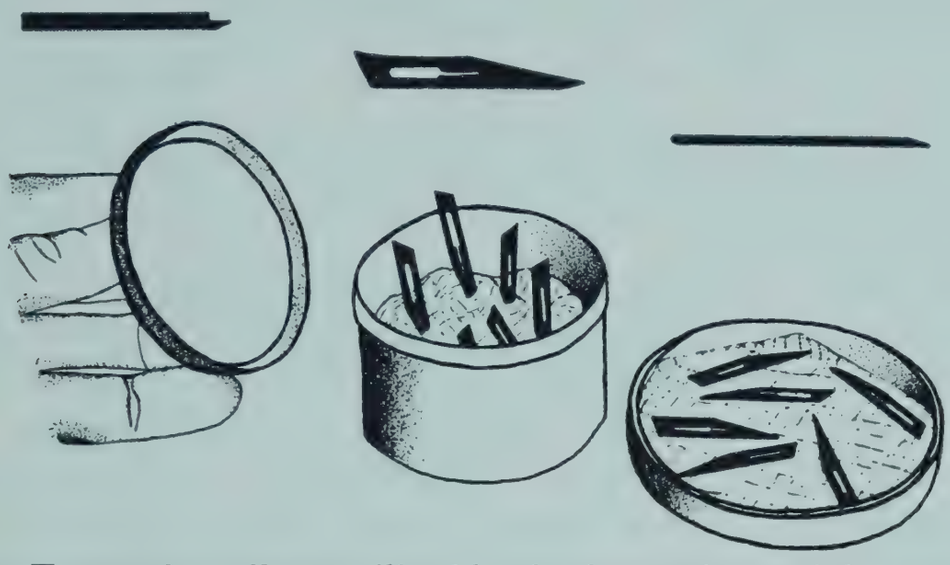


FIG. 1.2. Types of needles sterilized by dry heat using stainless steel ointment jar. Upper left: "Hemolet" needle. Upper middle: Bard-Parker blade (No. 11). Upper right: surgical needle (Hagedorn).

of glass tubing to protect the point and are sterilized by steam at 15 pounds pressure for 15 minutes in cotton stoppered glass test tubes (Fig. 1.1). Surgical blades and lancets, which are not rust proof, are best sterilized in dry heat at 160°C. for one hour in stainless steel ointment jars. The needles are stuck into a thick layer of cotton gauze to protect the points (Fig. 1.2). After removal of the jar from the heater, the lid should be left off until the metal cools to prevent precipitation of moisture.

"Hemolet" needles are heat sterilized in sealed envelopes. After use they may be discarded, or sharpened and sterilized by dry heat. They are particularly well-suited for the determination of the skin bleeding time for the projecting edge controls the depth of the puncture wound (Fig. 1.1).

Alcohol and other chemical disinfectants are inadequate for sterilization. Lancets stuck in a cork in a bottle of alcohol or wiped off with an alcohol sponge are not safe to use. If an autoclave is not available, instruments may be boiled for 15 minutes, but this technique does not assure inactivation of viruses.

The points of lancets are easily dulled or bent by dropping or by standing upright in glass containers. The sharpness of the point is best tested by examining under a magnifying glass or low power of the microscope. Lancets should be sharpened each day, using a smooth hard stone. Fine emory cloth is useful for removing rust.

The sites of choice for skin puncture are the end or side of the distal phalynx of the finger or the lobe of the ear. The finger is accessible and readily manipulated. The skin of the finger is relatively thick; but this is of some advantage, since the elastic skin tends to close the wound between collection procedures. Increased flow is readily obtained by producing venous stasis proximal to the site of puncture. The lobe of the ear is preferred by many because the skin in this area is thin, the puncture is less painful and post-puncture tenderness is avoided. When the patient is ambulatory and can sit on a stool so that the technician can approach the ear from the side and back, it is convenient to puncture the ear; but when patients are in bed or in wheel chairs or have respiratory or infectious diseases, it is awkward to have to lean over to get to the ear. If the skin wound is deep enough to get freely flowing blood, or if the patient has a hemorrhagic disease, it is more difficult to control the flow of blood from a puncture of the lobe of the ear than from a finger puncture (Figs. 1.3, 1.4, 1.5).

The heel, great toe or skin of the forearm below the elbow are the sites of choice for very young infants. Older children should be told what to expect when a puncture is to be made. To tell them that a needle stuck



FIG. 1.3. Method of puncturing the side of the middle finger to obtain blood. The ball of the finger can also be used. Note that the operator holds the finger tightly after having stroked it to promote circulation.



FIG. 1.4. Making light pressure around the puncture wound and showing the welling up of a drop of blood. The first drop should be wiped away, the finger surface completely dried so the drop will "stand" instead of flowing over the finger.



FIG. 1.5. Method of puncturing the lobe of the ear to obtain blood. This is said to be less painful than finger puncture.

into their skin will not hurt undermines their confidence and makes them much more difficult to manage later.

The site of puncture should be cleaned with 70 per cent alcohol on a cotton pledget or gauze sponge. The skin should be made tense previous to puncture. The puncture is usually made with a quick jab. Some prefer to place the needle point on the surface and to push the needle to the desired depth.

The first drop of blood should be wiped away and excess alcohol removed. If the finger is wet, the blood will spread along the surface, thus making collection difficult. Alcohol mixed with the blood distorts the cells and introduces a dilution error. In making thick drops for malaria, the alcohol fixes the cells and makes it impossible to properly lake the red cells. Pressure should not be made in the immediate neighborhood of the puncture, for this leads to dilution with tissue juices and favors clotting. If it is difficult to get sufficient blood for a given procedure, it is better to make a new puncture wound. Dull needles are the most frequent cause for not being able to obtain sufficient blood.

Venipuncture

Syringes. For the collection of blood for the usual tests, the 5-, 10- or 20-ml. syringes with metal tips and interchangeable barrels and plungers are recommended. The plunger in a good syringe will rotate freely in the barrel and yet will fit so snugly that air will not leak around the plunger when the tip is closed and the plunger drawn back. Syringes are expensive and easily broken; but it is economical to purchase syringes of the best grade, for they break less readily, are more accurately calibrated, the tip fits better in the needle hub and there are fewer failures in blood collection. A coating of mineral oil or white vaseline on plungers helps to prevent leakage in faulty syringes, but this treatment is not needed when good syringes are available. The greasing of syringes necessitates greater time in preparation, makes cleaning more difficult and introduces a foreign substance which interferes with some tests.

If syringes become stuck, the most simple method of loosening the barrel is to wrap a piece of rubber tubing tightly around the plunger and another piece of rubber tubing tightly around the barrel, thus enabling the glass to be firmly gripped for twisting. Various syringe openers, such as the "Syringe Opener B. D." and the "Syringe Piston Remover," Aloe, are available. They can be firmly attached to the stuck syringe and water forcefully ejected against the end of the plunger through the syringe tip. In using these syringe openers, the stuck syringe should be left hanging freely and a towel or other protector placed beneath so that the syringe will not break if forced off. If a needle becomes stuck to a syringe tip, the needle hub should be gripped with pliers and the syringe, not the needle, rotated.

It is necessary to use a sterile syringe as well as a sterile needle for each blood collection, for traces of blood left in the syringe tip may contain the virus of infectious hepatitis or other etiologic agents and this disease may be transmitted from one patient to another.

Wet syringes and needles cause hemolysis. Moisture is removed by aspirating air and expelling it repeatedly.

Tap water is sufficient for the washing of syringes which are being used for blood collection only, but syringes used for various treatment procedures should be washed with soap and water and scrubbed with a brush. They should be thoroughly rinsed to remove all traces of soap and the final rinse made with distilled water. Syringes must be thoroughly cleaned before sterilization. There are several detergents which are designed especially for removing traces of dried blood.

Syringes may be sterilized by boiling in a water bath for 15 minutes or longer, but this method is not recommended because it does not reliably prevent the transmission of virus hepatitis. Distilled water should be used, for tap water contains impurities which on drying become deposited on the glass and on the sides of the waterbath. Syringes should be assembled previous to boiling in order to minimize abrasion. It is preferred that all syringes be autoclaved. They should first be cleaned and dried, the plunger and barrel matched and the two parts wrapped separately and sterilized at 15–20 pounds pressure for 15 minutes. In some hospitals the dried syringes are assembled, placed in large test tubes with cotton stoppers and autoclaved. If dry heat is used, the syringes should be heated to 170°–200°C. for 30 minutes or longer. They can be stored in alcohol or other antiseptic solution, but complete sterilization cannot be assured by such solutions.

Needles. Stainless steel or rustless needles, one and one half inches long with moderately long bevels, are preferred. The gauge of the needles recommended for various procedures is as follows:

1. Small veins, small quantities of blood—21 to 23 gauge.
2. Routine collections—19 to 20 gauge.
3. 20 to 30 ml. blood—17 to 18 gauge.
4. 500 ml. blood for transfusion—14 to 16 gauge.

Small needles cause less pain and are easier to introduce than large ones, but the flow of blood through small lumens is slow and attempts to increase speed of flow by traction on the plunger leads to hemolysis of the cells (Fig. 1.6).

The Petroff needle, B.D., 19 gauge, $1\frac{1}{2}$ inches long, has a clip on the side for attaching to the test tube, thus eliminating the need for a syringe.

Needles should be inspected after use and sharpened if necessary. The needle is held at the desired angle and the bevel ground by a to and fro motion on a hard, smooth Arkansas stone. The rounded end that results when a cylinder is filed at an angle is brought to a point by turning

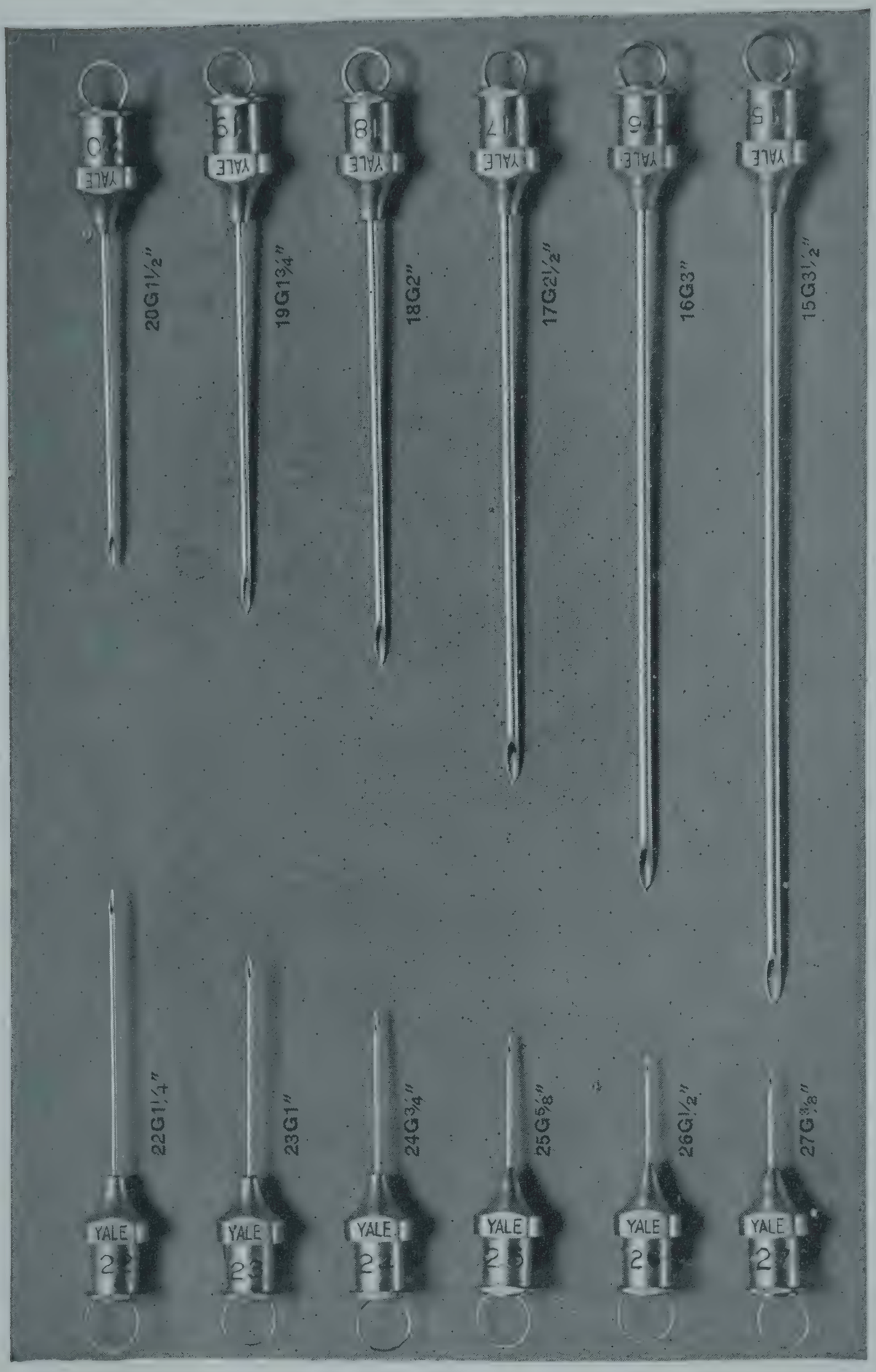


FIG. 1.6. Various types and sizes of needles used in laboratory work. Courtesy Becton-Dickinson Co.

the needle slightly and grinding down each side of the bevel. Needles should never be pointed on the side opposite the bevel, but beaks and burrs on this side of the bevel should be removed by grinding the needle flatly against the surface of the stone. Mechanically operated revolving stones are recommended for central supply rooms or units having to prepare numerous needles, but the use of this type of equipment requires special skill and is not satisfactory in the hands of inexperienced operators. Oil stones or coarse stones may be used for restoring badly sharpened needles or for changing the angle of bevel.

The stones used by many laboratories to sharpen needles may become deeply grooved. Needles passed through these grooves yield useable but inferior needle points. To restore the surface of a cutting stone, grind the surface on glass, using an abrasive such as "Electrolon ✻60." (Simonds Abrasive Co., Philadelphia). Rubbing one stone against another smoothes them both and takes away their abrasive qualities.

Debris and burrs at the bevel lumen may be removed by means of a small needle. A wisp of cotton held in a bayonet ear forceps or wrapped around the end of an applicator is used for cleaning the hub of the needle. Dried exudate or clotted blood may be loosened with mineral oil, by soaking in peroxide, soda bicarbonate solution, or by commercial detergents. All needles must be absolutely clean before sterilization. The patency of the lumen is tested by means of a stylet. Rust and stain can be removed by emory cloth. Metal polish may be used. Finally, the needles are washed in tap water and rinsed with alcohol and ether.

Needles may be sterilized by boiling in distilled water for 15 minutes but this method does not completely eliminate virus contamination. Care must be taken not to drop them in the sterilizer pan or to bang them against the edge of the pan when the hot water is drained off, because the points are easily bent or dulled. The needles may be taken directly from the sterilizer pan and attached to syringes or may be placed in alcohol, where they are kept until used. The alcohol is removed from the needle before the puncture is done, for alcohol along the needle tract causes pain and, if mixed with the blood, causes precipitation of proteins.

The best method of sterilization is by means of an autoclave. Needles to be autoclaved may be placed in tubes with constricted sides, which catch the hub and prevent the point from touching the bottom. If straight tubes are used, short pieces of glass tubing of small diameter may be used (Fig. 1.1). These tubes are stoppered with cotton. Needles may also be stuck through gauze or wrapped in gauze for autoclaving.

Some prefer to have stylets left in the lumen during sterilization procedures, thus assuring that the lumen is patent. The routine use of stylets greatly increases the labor of cleaning and the presence of a stylet favors the collection of moisture and corrosion of the metal.

It is a good policy to have the person who uses the needles responsible for their cleaning and sharpening, so that if there is failure in venipuncture because the needle is dull or the lumen is occluded, there is no alibi. If needles and syringes are centralized, they should be made the responsibility of one individual who gives personal attention to each needle.

Containers. Blood is often collected in test tubes of various sizes. For serological or chemical tests a 13 x 100 mm. tube without lip is often employed. If the tubes are to be transported or if there is to be a delay in examination, a stopper, preferably rubber, should be used. Wide mouth bottles, 15 to 30 ml. capacity, with cork stoppers or screw caps have the advantage over test tubes in that they do not require a rack, the blood mixes better with the anticoagulant and they are easier to fill, to get blood out of and to clean.

Sealed containers having a vacuum and with attached needles such as the "Keidel" or "Sheppard Blood Taking Tube" are available. The "Vacutainer, B. D." is a rubber stoppered vacuum tube, which is supplied with and without anticoagulants. A double pointed needle held by a plastic cylindrical cap is used. With these types of containers the vein is entered and the communication between the needle and the vacuum is established, thus enabling the collection of blood under negative pressure into a closed tube. The disadvantage of this type of equipment is the cost; the advantages are that they are convenient for occasional collections, reduce chance of contamination and are tightly sealed for transportation (Figs. 1.7, 1.8).

Arm Vein Puncture. In the performance of venipuncture, it is essential that the patient and the operator are both comfortable and that there is good light. The operator should be sure that he has a sharp needle of proper size and a dry syringe that works. Material for cleaning the arm, tourniquet, containers for placing blood and other equipment should be conveniently placed. If the patient is in bed, a towel or rubber sheet placed under the arm prevents soiling the linen.

When it is possible, the patient should be reclining when the puncture is performed, as he is more readily controlled in case he feels sick or faint. A short arm extension board helps. If the patient is seated, his arm should be extended over the corner of a table and placed on a towel or pad, or the patient may sit opposite the operator and extend the arm over a narrow, high table (34 to 36 inches from the floor).

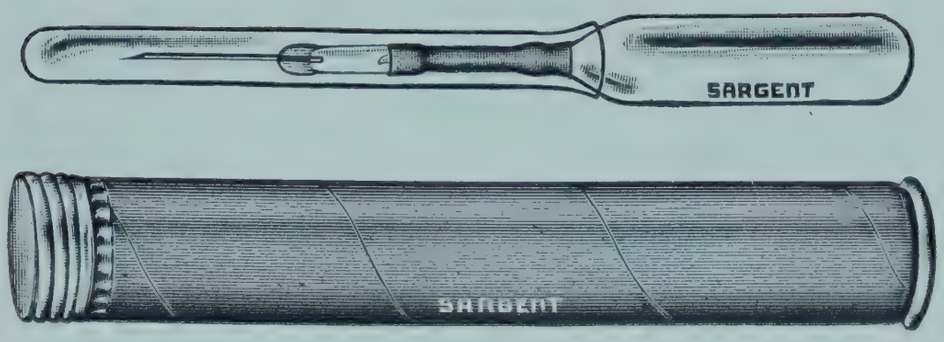


FIG. 1.7. The Keidel tube and mailing case. This consists of a vacuum bulb with a finely pointed glass tip to which is attached rubber tubing and needle, this being kept sterile with a small tube. A very satisfactory method for collecting and mailing blood samples. Courtesy E. H. Sargent Co.

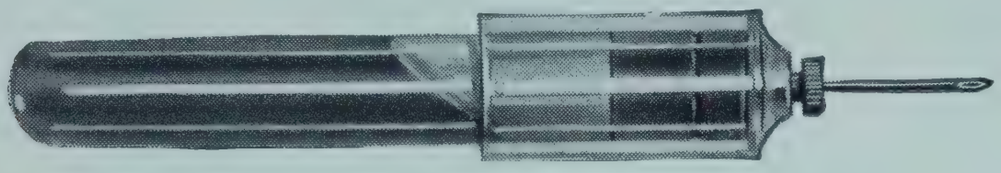


FIG. 1.8. Vacutainer. Courtesy Becton-Dickinson Co.

Have the patient roll up both sleeves in order to select the best vein. Be sure that there are no arm bands or that the sleeves are not so tight that they act as tourniquets. As a rule, the larger veins palpated in the anticubital region are more satisfactory than readily visible veins near the surface. Superficial veins are usually loosely fixed in subcutaneous tissue, tend to roll away from the needle, are easily collapsed and have thin walls. Do not select firm, cord-like veins, for these are usually thrombosed.

Devices to increase the amount of blood in the arm and to distend the veins are: 1) the placing of a tourniquet around the arm above the elbow; 2) having the patient open and close fist; 3) massaging arm; 4) slapping or thumbing the arm at the site of puncture; and 5) immersing the arm in warm water or covering with a hot, wet towel for 5 or more minutes previous to puncture.

The most readily available and quite satisfactory type of tourniquet is a piece of soft rubber tubing at least $\frac{1}{4}$ inch in diameter and 18 inches long. The tubing, slightly stretched, is placed around the arm and a loop tucked under for holding, so that it can be loosened by simply pulling the end. If a blood pressure cuff is employed, it should be inflated to 40 to 60 mm. of mercury.

The skin is cleaned with 70 per cent alcohol. For the collection of blood for culture, the alcohol should be preceded by iodine; but the routine use of iodine is not necessary. After cleaning the site, tighten the tourniquet and have the patient close his fist.

If the syringe is wet, be sure to dry it before attaching the needle.

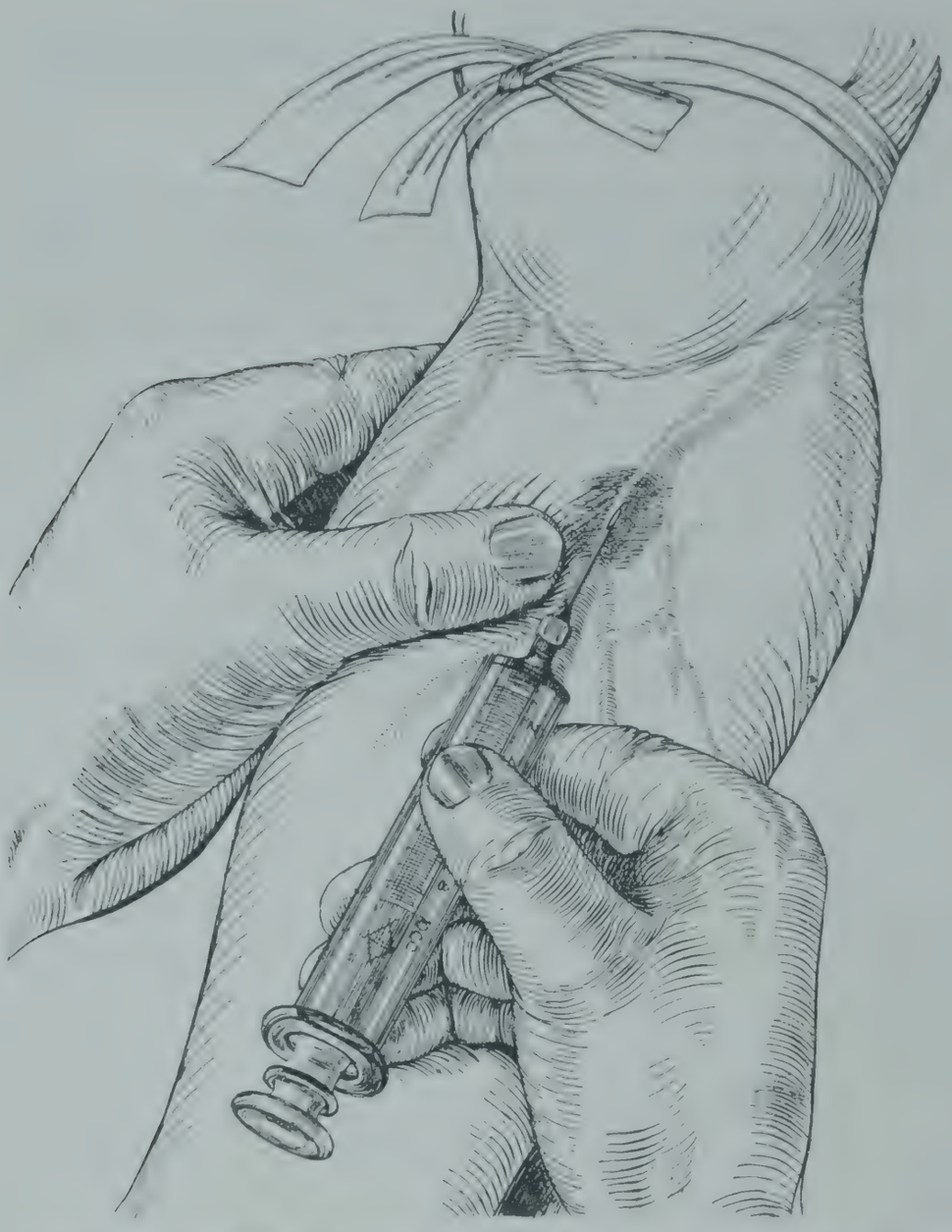


FIG. 1.9. Introducing needle into the vein for venepuncture. Note the bevel of the needle is upward. The syringe plunger is pushed in. The tourniquet is in place and the veins engorged.

Attach the needle tightly to the syringe. Test patency of the needle and syringe mechanism by aspirating and expelling air. Hold the syringe between the thumb and forefinger, using the other fingers as a steadying, directing prop. Hold the bevel of the needle up for large veins and down for small veins. Fix the skin below the site of puncture with the thumb of the other hand (Fig. 1.9). Hold the needle at an angle of approximately 45° and enter the skin several centimeters below the site of the intended vein entry. After going through the skin, lower the syringe and advance more nearly parallel to the vein until the lumen is entered. On entering the vein, aspirate by pulling the plunger back with the left hand (Fig. 1.10). Avoid forceful aspiration, for this causes the vein to collapse and causes gases to be suddenly removed from red cells, thus causing hemolysis. After sufficient blood has been removed,

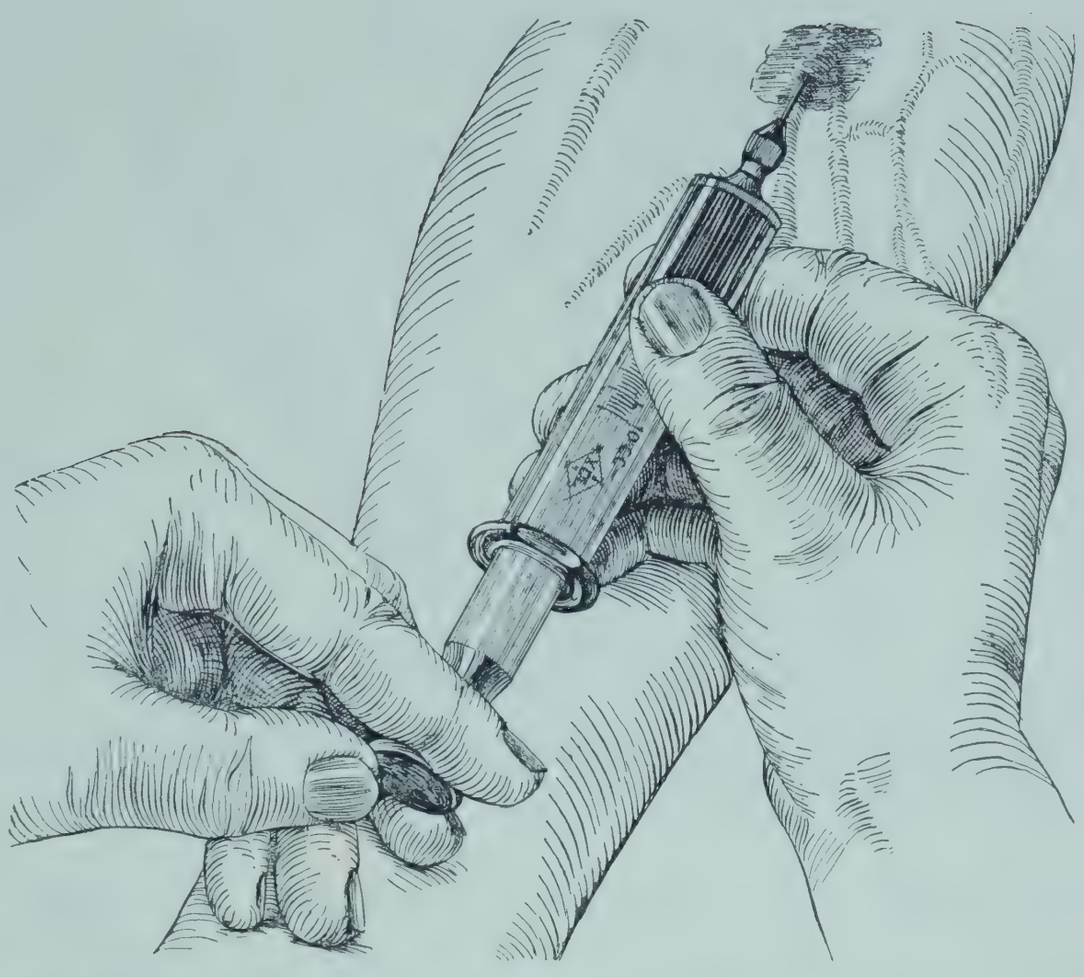


FIG. 1.10. Withdrawing blood from the vein. Syringe held firmly with right hand and left hand pulling plunger.

loosen the tourniquet, have the patient open his fist, place a pledget of dry cotton over the site of puncture and withdraw the needle (Fig. 1.11). Have the patient hold the pledget on firmly for several minutes. A bandage is not necessary.

Next remove the needle from the syringe and eject the blood carefully into a receptacle, allowing the blood to run down the inside of the container to avoid foaming (Fig. 1.12). If the needle is attached when the blood is ejected, it is likely to be shot off into the container. Forced ejection favors hemolysis.

After the blood is collected, rinse the syringe and needle with tap water. If water is not readily available, pull the syringe barrel partially out so that it will not be stuck when the blood clots and dries. Needles which cannot be cleaned immediately should be placed in a basin of water.

If there is difficulty in locating a vein or if a hematoma develops, loosen the tourniquet, remove the syringe and needle, replace the latter with a clean and unused needle and try the other arm. If the syringe has to be detached to collect a larger quantity of blood in another syringe or to start intravenous therapy, the flow of blood can be stopped and the



FIG. 1.11. Withdrawing needle from vein and application of pressure over puncture wound with an alcohol moistened cotton pledget. The tourniquet has been removed.



FIG. 1.12. Ejecting blood into a test tube. Note that the needle has been removed.

needle held in place by pressing on the vein at the level of the needle point.

Jugular Puncture. On babies, it is usually easier to get blood from the external jugular vein than from the anticubital veins. The child is

wrapped tightly in a sheet, which restrains the arms. The baby is placed near the end of a table and the head bent downward. The assistant firmly holds the child's body and head and makes light pressure above the clavicle. Crying increases the distension of the vein. A 20–21 gauge needle, $1\frac{1}{2}$ inches long, is used. It is directed toward the body.

Superior Saggital Sinus Puncture. The child is restrained by wrapping in a sheet. The head is held firmly, face up. The needle is inserted in the midline at a point just in front of the junction of the parietal bones and is directed toward the occiput. Puncture in this area should be resorted to only in emergencies. Medication should never be given by this route.

Femoral Vein Puncture. Wrap the child tightly in a sheet, which restricts the arms. Feel for pulsation of the femoral artery and keep finger on the artery while a needle attached to a syringe is inserted just medial to the artery below Poupart's ligament. A slight negative pressure in the syringe allows the blood to appear as soon as the vein is entered.

Arterial Puncture. Occasionally it is impossible to collect blood from individuals due to poor arm veins. In such instances, arterial puncture can be attempted. Culture of arterial blood will sometimes reveal positive findings in subacute bacterial endocarditis when venous blood is negative.

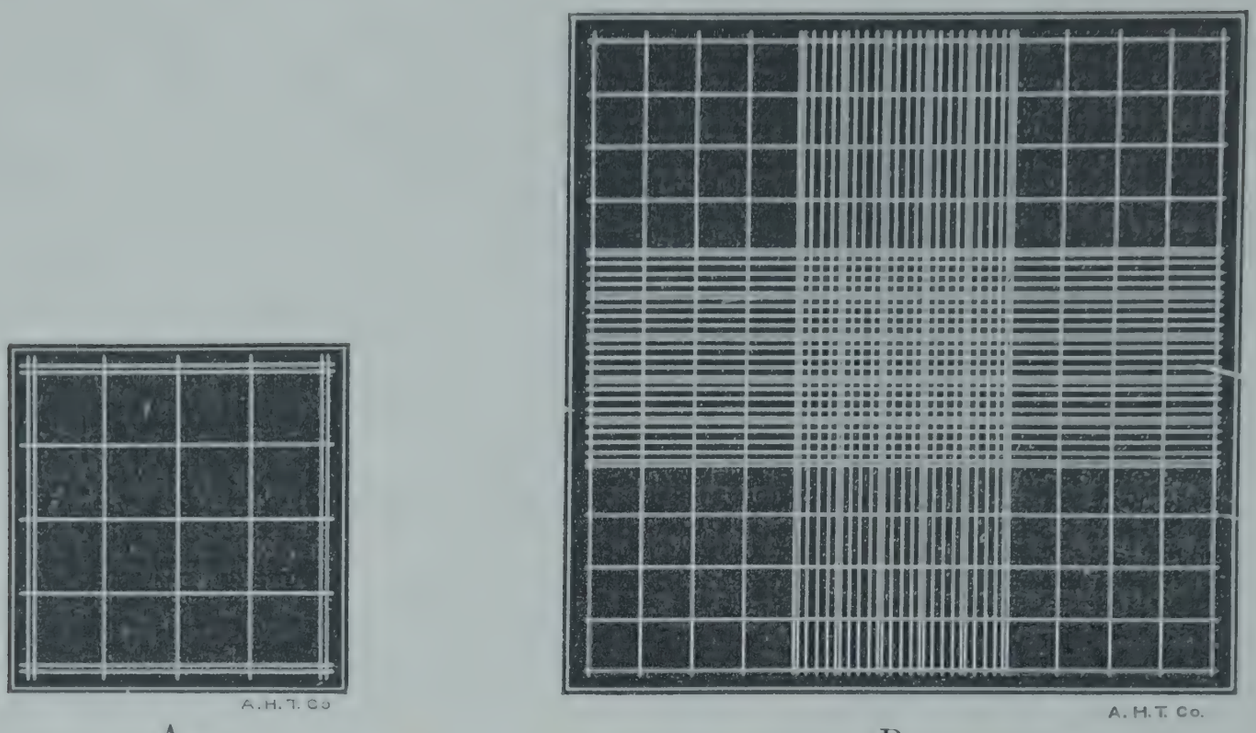
The needle used should be relatively small, 20 to 23 gauge, with a medium bevel, $1\frac{1}{4}$ inches long and very sharp. The brachial artery where it lies superficially on the anterior flexure of the elbow is the operative site of choice. The radial artery of the wrist may also be used. No tourniquet is needed. The arm is hyperextended by use of a small pad under the elbow. The syringe is held at an angle of about 45° until the skin is punctured and then the needle is advanced nearly parallel to the artery. On entering the artery, there is some pain. The force of the blood will push the plunger up spontaneously. After collection of sufficient blood, remove the needle, apply a sponge forcefully and bandage.

Intravenous fluids must not be given into arteries, as thrombosis of terminal vessels and gangrene may result.

BLOOD CELL COUNTING

Equipment

Counting Chambers. Numerous types of counting chambers with various types of markings have been designed. The type which is most



A B

FIG. 1.13. A. Group of sixteen smallest squares of Improved Neubauer Ruling, showing transparent boundary enclosed between two engraved furrows. B. Entire area of Improved Neubauer Ruling.

widely used at the present time is one made of a single piece of colorless glass with two separate elevated counting platforms, each having the improved Neubauer ruling (Fig. 1.13). The platform on which the engraved lines are placed is surrounded by a moat. On each side of the platform there is an elevated glass support on which the coverglass is placed (Fig. 1.14). The distance between the counting surface and the bottom of the coverglass is 0.1 mm. Diluted blood is introduced by capillary attraction between the counting platform and the coverglass.

The improved Neubauer type of ruling consists of a system of squares. There is a square measuring 3 x 3 mm. (9 sq. mm.) divided into 9 squares, each 1 x 1 mm. (1 sq. mm.). The four corner squares, which are used for the white cell count, are divided into 16 intermediate squares, measuring 0.25 x 0.25 mm. (Fig. 1.30 ABCD). The central square millimeter is redivided into 25 medium sized squares, each one of which measures 0.2 x 0.2 mm. (Fig. 1.30 EFGH). Each one of these medium-sized squares is redivided into 16 small squares which measure 0.05 x 0.05 mm. The total number of the small squares in the central ruled area is 400. As a rule, 5 of the medium sized squares (80 small squares) are used for the erythrocyte count. The ruling in most counting chambers is so designed that the medium squares of 16 small squares each are enclosed by a translucent boundary included between two engraved lines. In some counting chambers triple lines are used and the central line constitutes the boundary.

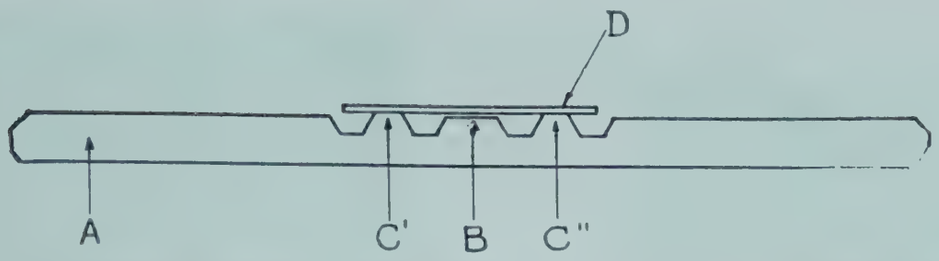


FIG. 1.14. Counting chamber. Side view. *A*, Longitudinal section of chamber. *B*, ruled area. *C'*, *C''*, Supports for coverslip. *D*, coverslip. Note space between *B*, and *D*.

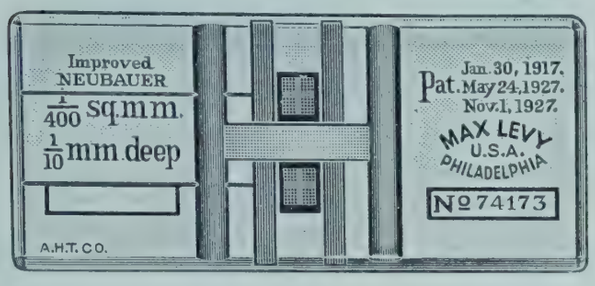


FIG. 1.15

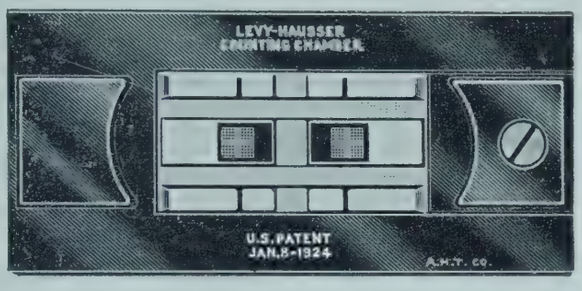


FIG. 1.17



Fig. 1.16

FIG. 1.15. Levy Counting Chamber with Improved Neubauer double ruling.
FIG. 1.16. Hemacytometer consisting of a red cell pipette, a white cell pipette and a counting chamber enclosed in a velvet lined case.
FIG. 1.17. A counting chamber mounted in an unbreakable bakelite holder.

The newer Levy counting chambers have an opaque incline on each side of the ruled area which aids in finding the ruled area, as well as in filling the chamber and minimizes the chance of introducing bubbles or overcharging. A prefocusing matte and finding lines leading to the ruled area are other desirable features of this instrument.

The Levy-Hausser counting chamber (Fig. 1.17) consists of a counting chamber mounted in a moulded Bakelite holder which minimizes the chance of breakage, and which protects the under surface of the glass slide from scratching.

The Levy quadruple counting chamber has a standard depth of 0.1 mm., two ruled areas for white cell counts and two ruled areas for erythrocyte counts. The Fuchs-Rosenthal ruling (Fig. 1.18) is used in the white cell counting area and the improved Neubauer ruling in the red cell counting area. This instrument is used for studies in which duplicate counts are indicated.

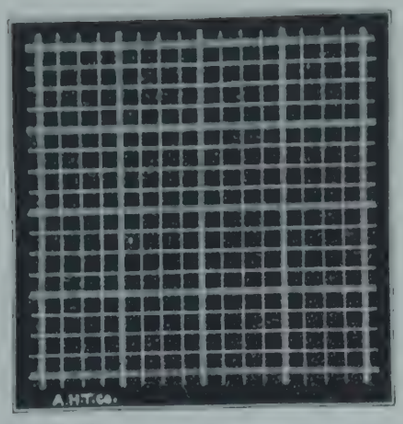
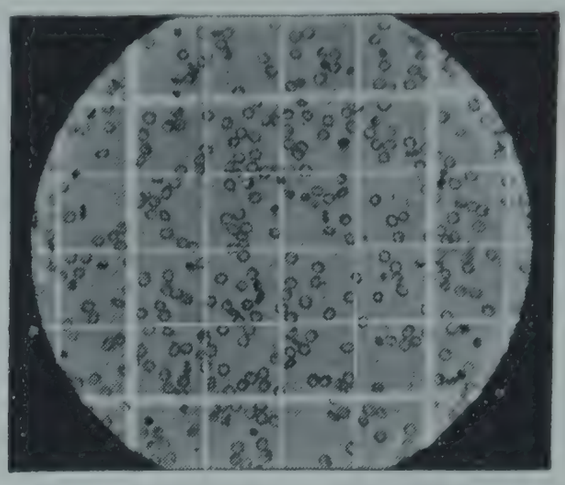


FIG. 1.18. Fuchs-Rosenthal Ruling.



The ruled area of the Spencer bright line counting chamber. Courtesy Spencer Lens Company.

The Levy counting chamber with double or quadruple ruling and the Levy-Hausser counting chambers are made with colorless or green glass. The green glass is preferred by many because it gives a subdued background, the etched lines are sharply defined and eye fatigue is lessened.

The Spencer "Bright-Line" hemocytometer and the Clay-Adams "Hy-Lite" chamber have a transparent layer of metal fused on the surface of the glass. Lines ruled through this metal stand out as bright lines against a gray background in which there is a minimum of glare.

The Hellige "Eureka" counting chamber has a flashed red lower surface except in the two areas below the ruled surface, thus making it easy to find the area used for counting.

For counts on cerebrospinal fluid and other fluids in which there are relatively few cells, the Levy counting chamber with Fuchs-Rosenthal ruling is sometimes used. This counting chamber has a depth of 0.2 mm. The ruling consists of 16 one-millimeter squares, bounded by triple lines. Each square is subdivided into 16 smaller squares. The cells in the entire ruled area (16 sq. mm.) are counted. This number divided by 16 gives the number per square millimeter which is multiplied by the dilution factor and by 5 to give the number of cells per cubic millimeter.

For research work, in which maximal accuracy is desired, counting chambers, coverglasses and pipettes which meet the U. S. Bureau of Standards, specifications are used. Instruments that have been certified by the U. S. Bureau of Standards are etched with a seal, consisting of a circle containing the letters U. S. and the year the test was made. For routine clinical work, U. S. certified instruments are not needed, provided the instruments are purchased from manufacturers whose record as makers of instruments of precision has been established. Doctors

should be careful not to purchase blood counting instruments from undetermined sources and should be suspicious whenever bargains are offered, because such instruments are often defective.

Coverglasses specially made for cell counting should be used. Ordinary coverglasses such as are used for blood smears are not satisfactory, for they are not rigid enough, do not have sufficiently plane surfaces and may have visible defects.

Counting chambers and coverglasses should be cleaned immediately after use by washing in tap water and drying with a clean lint-free cloth. Soap and warm water, soda bicarbonate solution or ether may be used to remove grease or dried fluids, but strong acids or alkalies should not be used. Gauze or linen should not be used for drying, for these materials will scratch the ruled areas. Fingerprints on the surfaces are difficult to remove and lead to errors in counting; therefore always handle the counting chambers and coverslips by the edges.

Diluting Pipettes. For diluting blood and other fluids the Thoma type of glass pipette is most widely used. This pipette consists of a graduated capillary tube having a volume of one unit with calibrated markings at 0.1 unit. Above the capillary tube is a mixing bulb, containing a glass bead. Above this bulb is a capillary tube with an engraved mark. The white cell pipette has a white bead and the marking above the bulb is "11"; the red cell pipette has a red bead and a marking above the bulb "101" (Fig. 1.19).

In making a white cell count, blood is drawn up to the 0.5 mark and diluting fluid to the 11 mark. The last diluting fluid that enters the lower capillary tube does not enter into the dilution of blood within the bulb. This diluting fluid in the graduated capillary tube is discarded before the count is made. Although the diluted blood is drawn to the 11 mark, the real dilution is 0.5 to 10, or 1 to 20. In making a red cell count the blood is drawn to the 0.5 mark and diluting fluid to the 101 mark. Since the last diluting fluid does not mix with the blood in the bulb and is discarded before the chamber is charged, the actual dilution is 0.5 to 100, or 1 to 200.

If very low counts are suspected, the blood may be drawn to the 1.0 mark instead of the 0.5 mark, thus giving a 1 to 10 dilution for a white cell count and a 1 to 100 dilution for a red cell count. If there is insufficient blood to reach the 0.5 mark, the sample can be taken at any mark below 0.5 and suitable correction made for the increased dilution.

The Trenner automatic diluting pipette is made by fusing a separately made bulb on the end of a piece of straight capillary tubing, the upper

end of which terminates abruptly in a ground and polished surface at right angles to the longitudinal axis (Fig. 1.19). The Trenner pipette used for white cell counts has a white glass bead and a "21" mark above the bulb. The volume of the capillary tubing is exactly $\frac{1}{20}$ of the total volume of the bulb. The Trenner automatic pipette for red cell counting is larger than the white cell pipette, has a red glass bead and a mark "201" in the capillary tube above the bulb. The capillary tubing has a volume exactly $\frac{1}{200}$ of the volume of the bulb. The advantages of the Trenner pipettes are that the capillary tube is filled by capillary attraction and the blood automatically stops at the end of the tube, providing

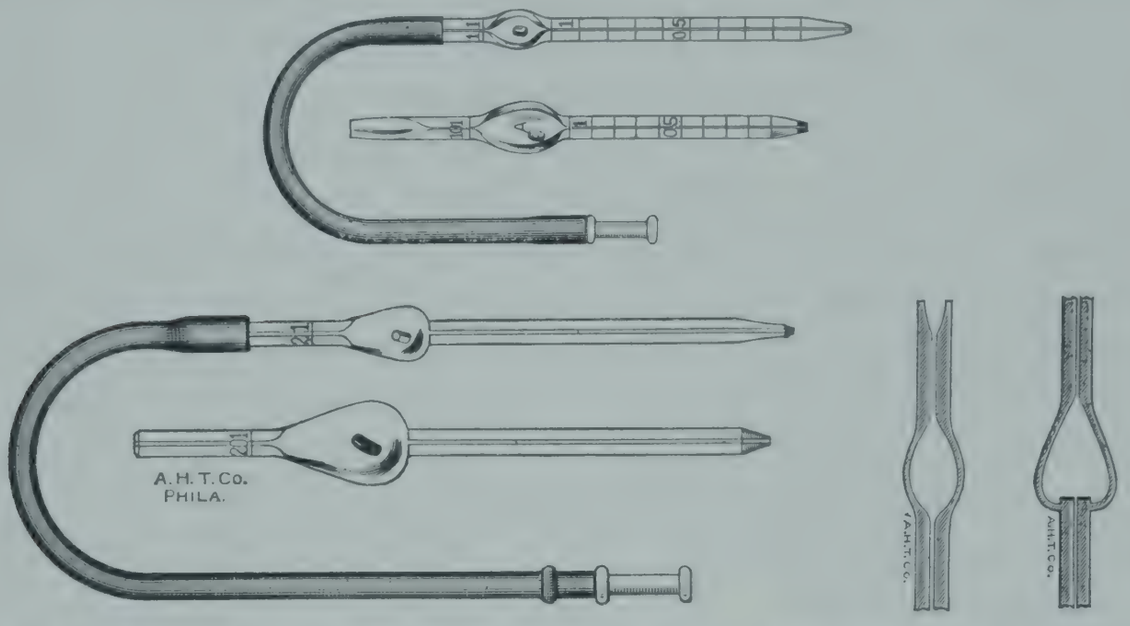


FIG. 1.19. Various diluting pipettes for counting blood cells. Above, the Thoma pipettes; left, Trenner automatic pipettes; and right, cross sections of the Thoma and Trenner pipettes.

accurate control of the blood column. Disadvantages are the increased cost and the greater fragility of the instrument.

The rubber tubing which is attached to the blood diluting pipette should be at least 10 inches long, so that the pipette is far enough from the eye to enable one to read the graduation marks. The tubing should have a sufficiently heavy wall to resist collapse during suction. The mouthpieces which are used on the ends of the rubber tubing may be detached after use, washed and stored in alcohol. Some workers prefer to detach the rubber tubing without removing the mouthpieces and to leave the mouthpiece ends of the tubing immersed in a beaker of alcohol. A 25 gauge hypodermic needle used as a mouthpiece makes an excellent throttle for filling a pipette accurately.

Dirty pipettes should first be washed with water followed by acetone or 95 per cent alcohol using a water suction pump. Multiple aspirating



FIG. 1.20. Pipette containing diluted blood with rubber band stretched over the ends to prevent escape of fluid.

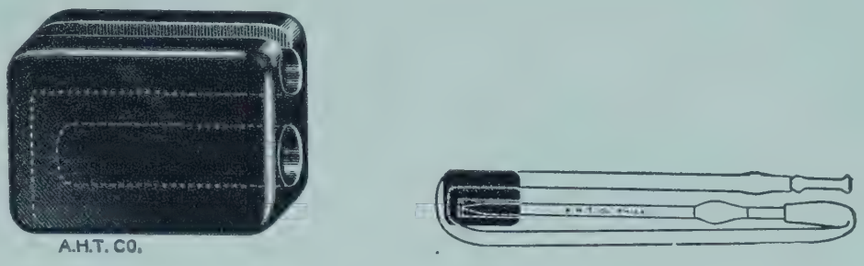


FIG. 1.21. A satisfactory type of pipette closure. Left. Actual size. Right. Showing closure in position on Thoma pipette, with pipette automatically sealed by continued tension of the stretched rubber tubing.

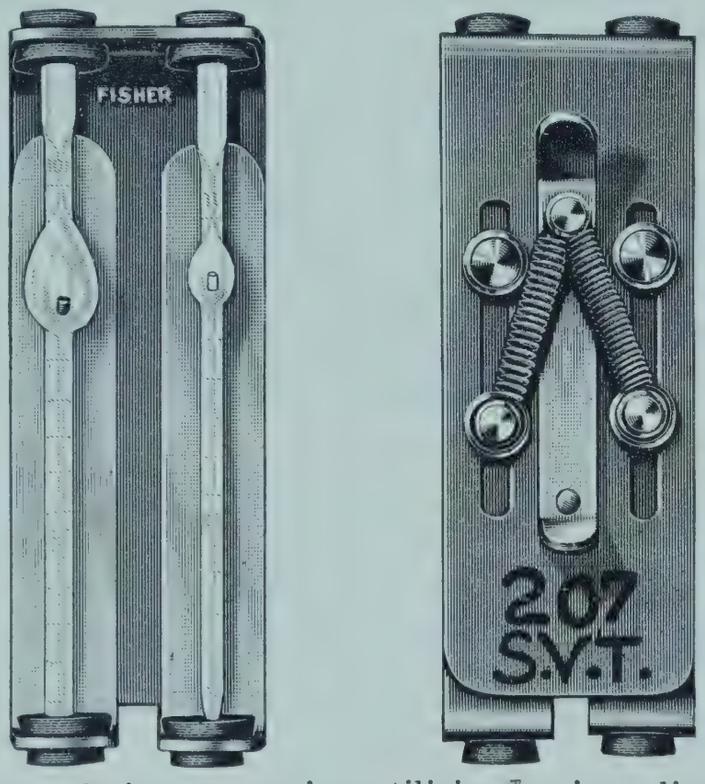


FIG. 1.22. A type of pipette carrier utilizing spring clips. Left. Front—pipettes are held and sealed by spring tension. Right. Rear—finger grips for shaking, and a place to mark. Courtesy Fisher Scientific Co.

nozzles or Haden cleansing apparatus are available for cleaning and drying many pipettes at the same time (Fig. 1.23). If mouth suction is used, it is advantageous to employ ether as a final rinsing and drying agent; but ether is not necessary when mechanical suction devices are available. After cleaning, air is drawn through the pipette until all moisture is removed and the glass bead is freely moveable. Do not blow through the pipette to dry, for moisture from the breath will condense in the glass bulb. If pipettes cannot be cleaned immediately after use,

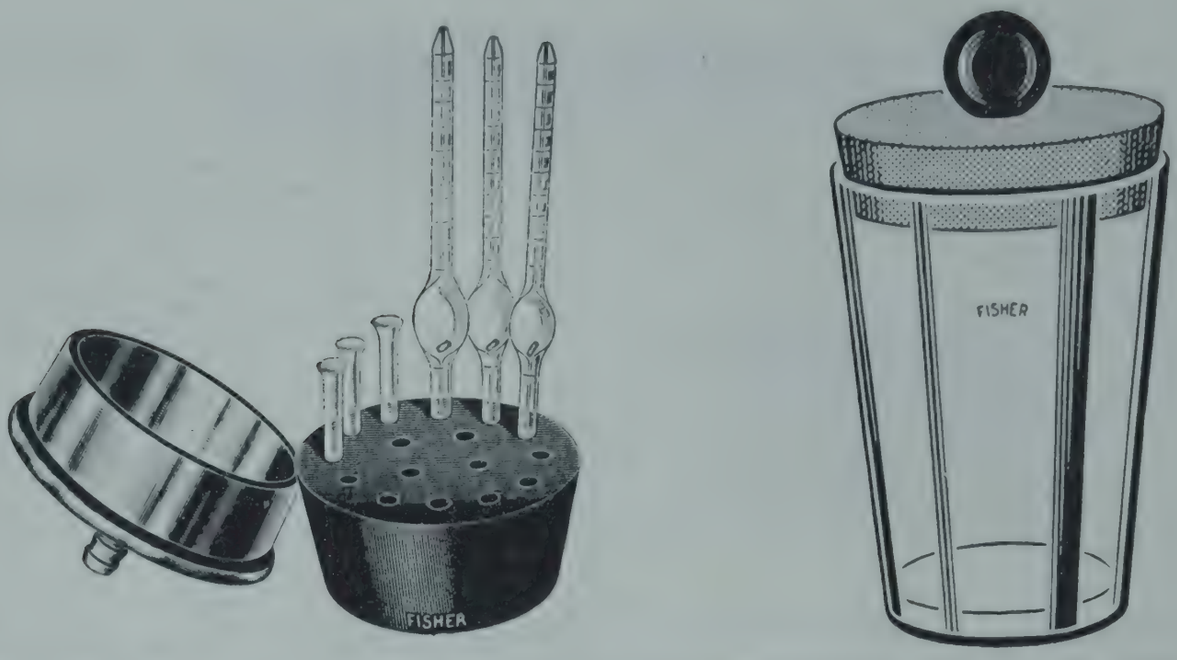


FIG. 1.23. Pipette cleaner and pipette wash jar, for washing and drying diluting pipettes (Courtesy of Fisher Scientific Co.).

use, leave them upright in a glass of water to prevent diluting fluid or blood from drying at the tip. Debris may be dislodged from capillary tubing by means of a horse hair or small wire which can be purchased for this purpose. Pipettes that are stained or plugged with clotted blood may be cleaned by leaving to soak in nitric or sulfuric acid, sodium hydroxide solution or sodium hypochlorite (Chlorox). Strong acids should not be drawn suddenly into wet pipettes for sufficient heat is generated to cause dangerous explosions.

Various devices are available for closing the ends of pipettes so that they can be transported without escape of fluid. The rubber tubing attached to the pipette may be wrapped around the ends and tied in a knot. Wide rubber bands (Fig. 1.20), bakelite pipette holders (Fig. 1.21), and metal racks with spring clamps (Fig. 1.22) are also employed.

Bottles for Holding Blood Counting Fluids. A convenient set of bottles for holding all fluids necessary for making blood counts and for cleaning pipettes may be arranged in a single row by binding together with adhesive tape, allowing enough clear space at the top for visibility. Designation of the contents of each may be printed upon the tape. Short bottles of clear glass are to be preferred to tall, colored ones, since the immersed pipette can be better observed while filling.

It is preferable to use small containers and to replace the contents with fresh fluid frequently, thereby avoiding the accumulation of debris and cells from previous counts and the growth of fungi.

Trays for Blood Collection. When collecting blood in the hospital or in the home, it is necessary to have a tray or box to transport equipment. Various types of trays are available or can be made (Figs. 1.24 and 1.25).

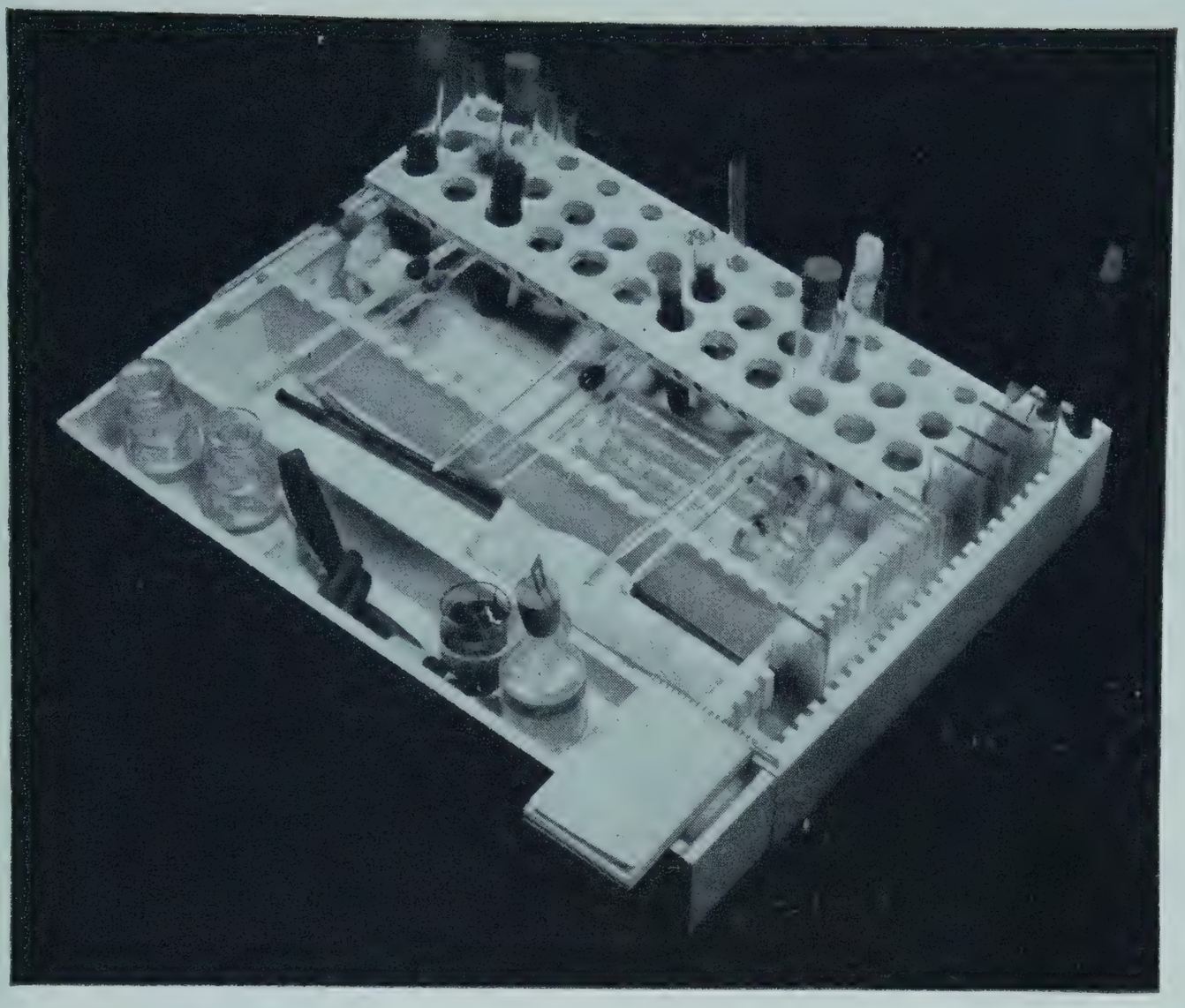


FIG. 1.24. A suitable tray for hematologic work constructed of light wood, size approximately 12 x 14 inches. Bottles for alcohol and diluting fluids and tourniquet can be placed in first compartment, sterile wrapped syringes in second compartment, dirty syringes and soiled cotton on third compartment. Also note rack for holding pipettes and sterile needles in cotton plugged tubes. The holes are perforated in three different sizes. Slide slots are at one end in which two slides are placed back to back. Designed by Clara M. Becton, St. Johns' Hospital, Tulsa, Okla. Courtesy J. B. Lippincott Company, from Diseases of the Blood, by Kracke and Garver, 1937.

Leukocyte Counting

Principle. Blood is diluted in a white cell pipette with an acid solution which lakes the red cells. This solution does not destroy the white cells, which are counted in a hemocytometer. Stains added to the diluting fluid aid in visualization of the nuclei.

Diluting Fluids. The most commonly used diluting fluids for white blood cells are:

1. Glacial acetic acid, 2 ml.
   Gentian violet (1 per cent aqueous), 1 ml.
   Distilled water, 100 ml.

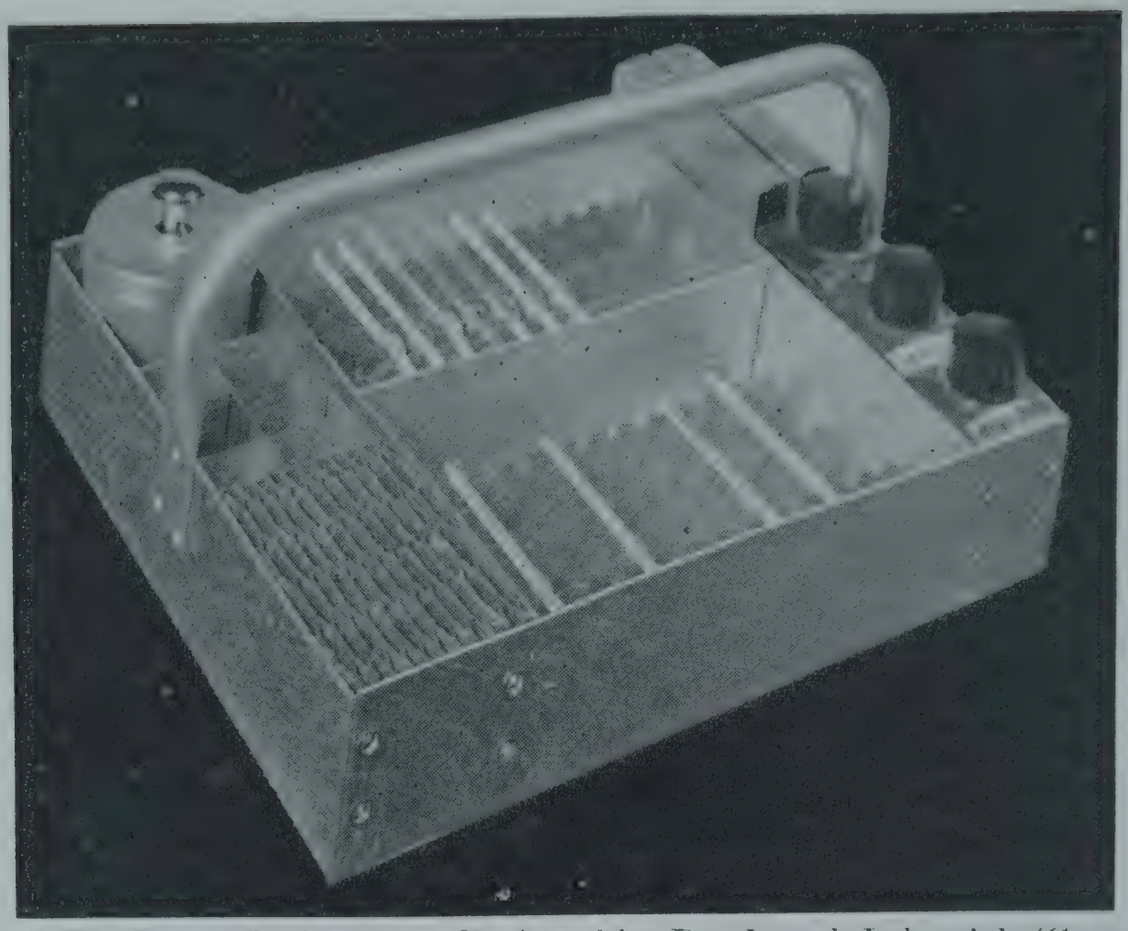


FIG. 1.25. Hematologic tray. Designed by Dr. Joseph Lebowich (Courtesy of The Sargent Company, Cedar Rapids, Iowa).

2. A somewhat similar solution may be quickly prepared as follows:
   Glacial acetic acid, 4 drops.
   Distilled water, 20 ml.
   Add methyl violet or gentian violet, just enough dry crystal to slightly color the solution.
3. N/10 Hydrochloric acid.
   One milliliter of concentrated HCl in 100 ml. of water is approximately tenth normal and is sufficiently accurate for this test. This diluting fluid is preferred when the clinical model of the Haden-Hausser hemoglobinometer is used because the white cell count and the hemoglobin estimation can be done with the contents of the same white cell pipette.

Filling the Pipette. The blood may be drawn from a freely flowing skin puncture wound or from a well-mixed oxalated blood specimen. Hold the pipette horizontally at nearly right angles to the line of vision so that the exact height of the column may be seen. The side of the tip should rest against the skin, but the end must be free (Fig. 1.26). If the drop of blood is too small or the tip is not kept immersed, air bubbles will enter with the blood and cause a discrepancy in the count. Should the column of blood be drawn slightly above the desired mark, it may be

lowered by blowing out or touching the tip of the pipette to the finger or piece of filter paper.

Wipe excess blood from the tip and draw diluting fluid to the 11 mark. While the diluting fluid is being drawn up, the pipette should be revolved, the glass bead kept in motion and air bubbles prevented from adhering to it or to the wall of the bulb (Fig. 1.27).

The tip of the pipette is now closed with the thumb or finger and the tubing removed. The pipette is shaken a few seconds and placed in a

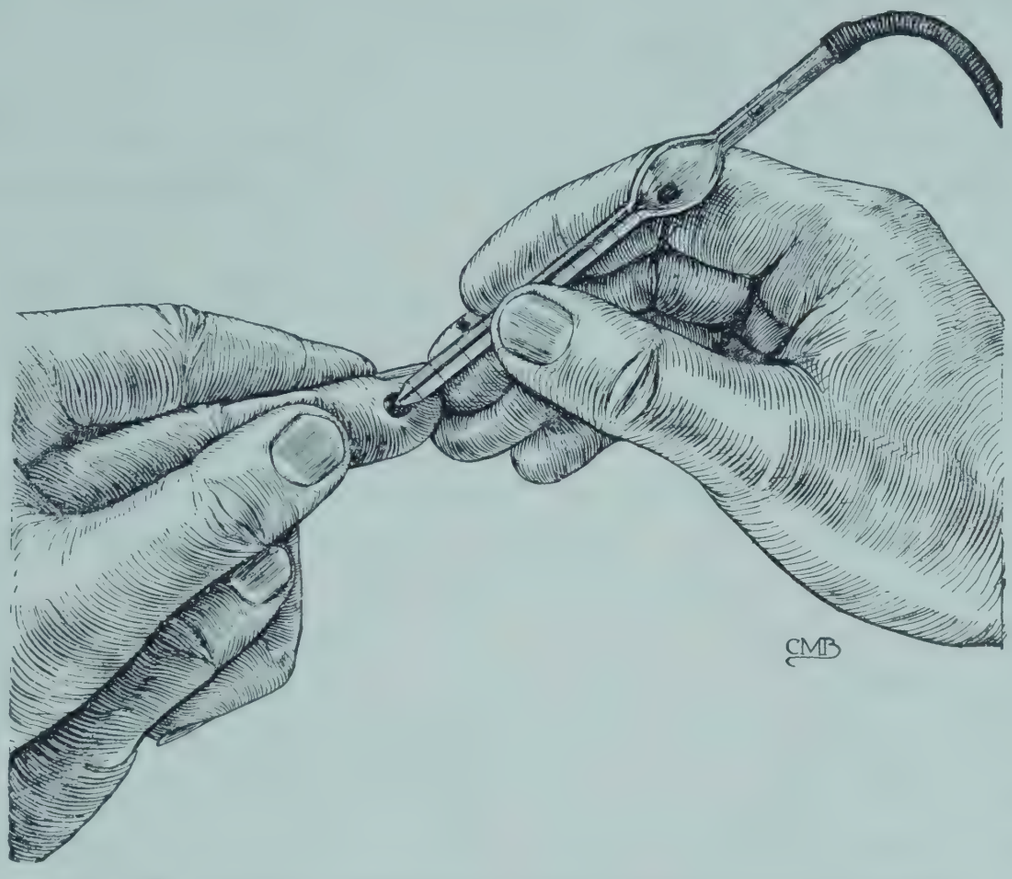


FIG. 1.26. Proper method for filling the blood cell pipettes

rack in a horizontal position, which prevents the diluted blood from running out. It also keeps the ends of the pipette from touching any object, which would cause fluid to escape by capillary attraction.

In laboratories performing tests on oxalated blood or collecting blood in the same room in which the counts are made, the filled pipettes can be placed on the sheet of paper used for the patient's report. The pipette may be stuck through the laboratory request sheet, thus identifying the pipette, keeping it from rolling, holding it in the proper horizontal position and keeping the ends from touching any neighboring object.

Counts should be done soon after collection, but if this is not possible the diluted blood should be placed in a refrigerator, where the cells will

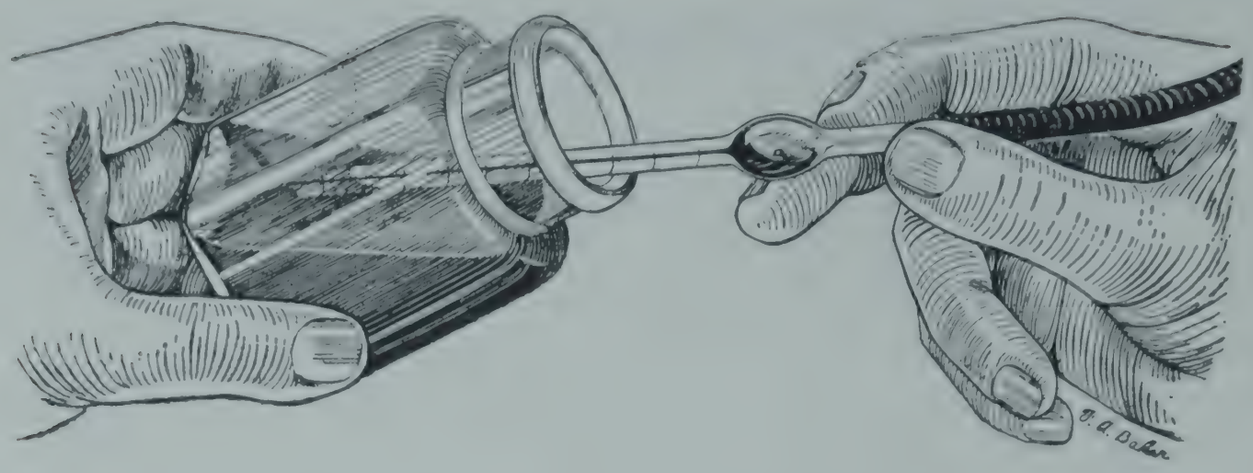


FIG. 1.27. Filling pipette with diluting fluid

remain well preserved for hours. The pipette should never be left in the vertical position, for the cells will settle into the capillary tubing and give an abnormally low count.

Shaking. Pipettes should be shaken vigorously just before the counting chamber is charged. Mechanical shakers of various kinds are available, but hand shaking is preferred by most technicians. The majority of workers remove the tubing previous to shaking, but the pipette may be shaken with the tip closed and the tubing kinked over the other end. During shaking, the pipette is held horizontally and shaken in a to-and-fro rotary motion for 2–3 minutes.

Charging the Counting Chamber. Absolute cleanliness and freedom from grease of both the counting chamber and coverglass are essential. Care must be taken that there are no lint or particles of dust and that the coverglass rests evenly on the supporting platforms. After the pipette is shaken thoroughly, the first few drops are discarded by resting the pipette tip on a piece of absorbent material. Then the pipette tip is touched to the side of the counting chamber and the fluid allowed to run in (Fig. 1.28). Some workers prefer to leave the tubing attached and control the flow into the chamber by pressure on the tubing or by mouth pressure, but the majority remove the tubing and control the flow of blood by means of a finger on the end of the pipette, as in standard chemical procedures. Care must be taken that a drop of fluid does not form at the end of the pipette before the edge of the coverslip is touched, for this will lead to flooding the moat. If the coverglass is floated off or fluid escapes into the trenches on the side of the counting platform, clean the chamber and repeat the procedure.

Counting. Let the cells settle for 1–2 minutes so that all the cells are in the same plane. Using the low power objective, locate the ruled area. Find the upper left large corner square (1 sq. mm.) which is divided into 16 intermediate squares. Adjust the mirror, diaphragm

and substage distance so that the nuclei and marking lines are clearly seen. Starting at the upper left hand corner and using a system of meander as shown in Fig. 1.29, count the cells in each of the 16 intermediate squares. In order to avoid counting the same cells twice and in order to correctly count those cells that touch the outer boundary lines, count in any given square those cells that touch the left and upper boundary lines and ignore those cells that touch the right and lower boundary lines (Fig. 1.29). Record the total leukocytes counted. Then move the counting chamber so that the upper right square millimeter

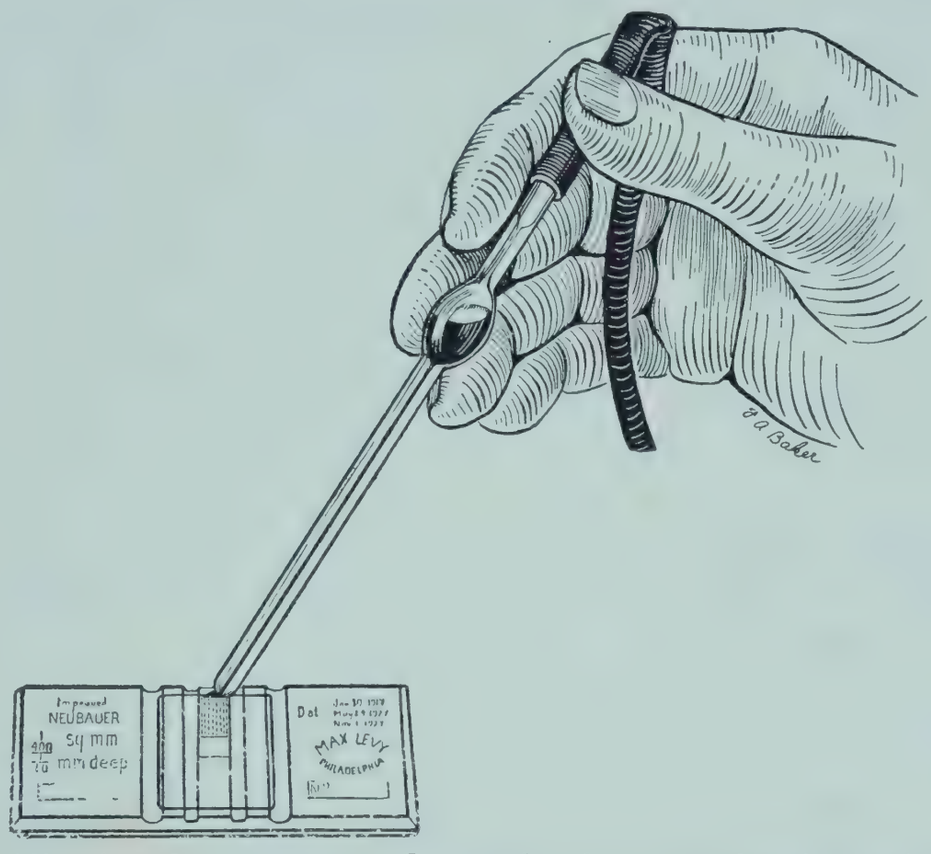


FIG. 1.28. Allowing the contents to flow under the cover glass by capillary attraction after they have been thoroughly mixed in the bulb of the pipette and the first drop expelled.

(Fig. 1.30 ABCD) is visible. After counting the white cells in this area, count in succession those in the lower right and in the lower left corner squares, making a total of 4 square millimeters counted.

Calculation. Since the cells in 4 square millimeters have been counted and the dilution is 1:20 and the distance between the counting platform and the coverslip is $\frac{1}{10}$ mm. the number of cells per cubic millimeter is calculated by the following formula:

$$\frac{\text{Total leukocytes in 4 sq. mm.}}{4} \times 20 \times 10 = \text{number of leukocytes in 1 cu. mm.}$$

or

Leukocytes in 4 sq. mm. $\times$ 50 = number of leukocytes in 1 cubic millimeter.

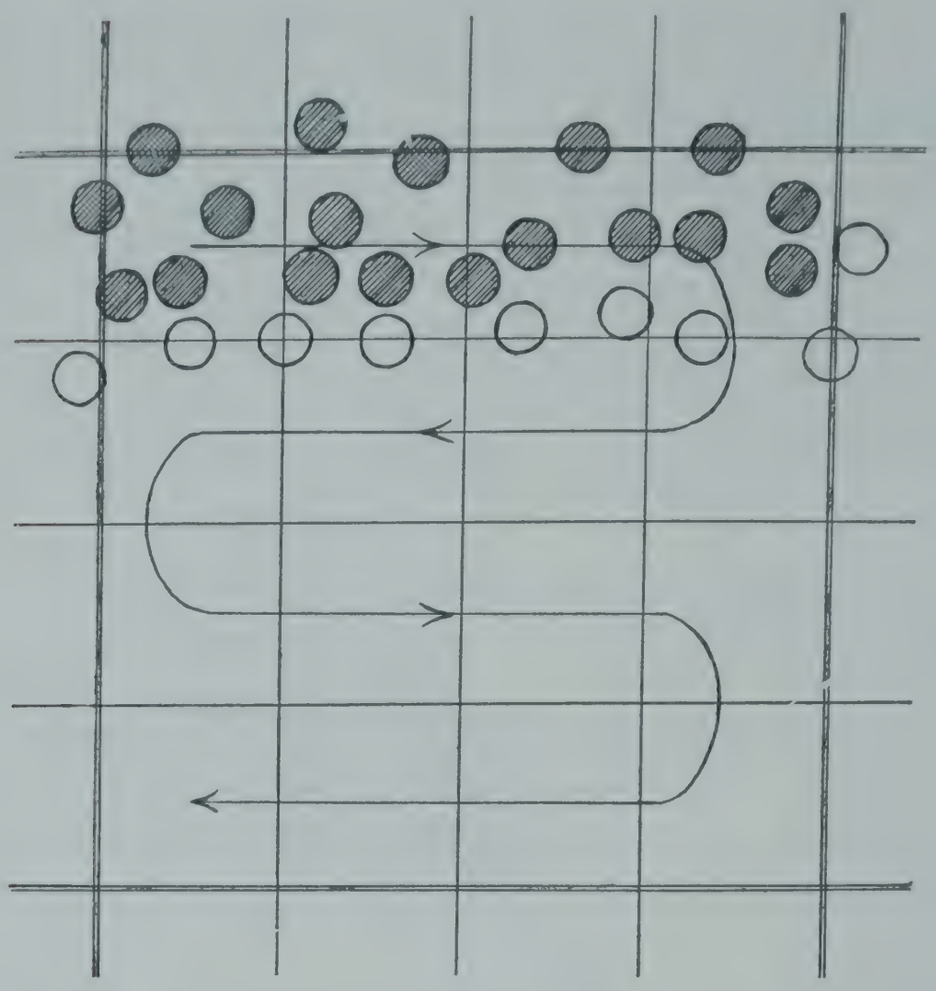


FIG. 1.29. Showing manner of counting red cells. The arrow indicates the direction in which count should be made. Eighteen shaded cells are counted in the first line of four small squares.

Example:

Square No.	Total Leukocytes
1	35
2	40
3	38
4	37
Total	150 × 50 = 7,500 leukocytes per cu. mm.

Normal Values. The leukocyte count varies between 5,000 and 10,000 per cubic millimeter. Values above 10,000 per cubic millimeter are designated as leukocytosis and below 4,000 as leukopenia.

The error in white cell counting methods is ± 10 %.

Nucleated red cells cannot be distinguished from lymphocytes in the white cell counting method and correction of the white cell count should therefore be made when there are numerous nucleated red cells.

For further discussion of white cell counts see Chapter V.

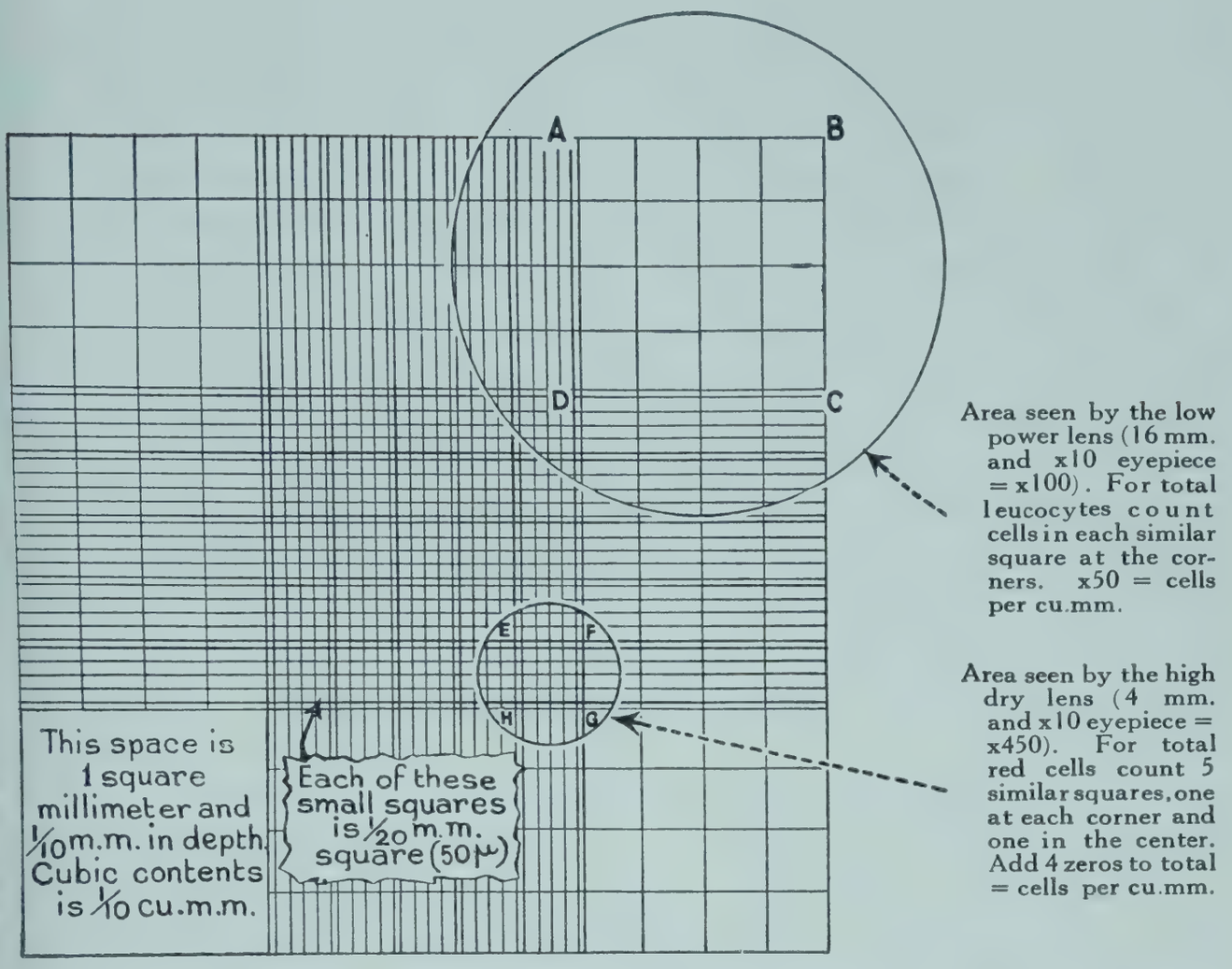


FIG. 1.30. Improved Neubauer ruling on a hemocytometer. After Nicholson Laboratory Medicine, 1934. Lea and Febiger.

Eosinophil Counting

Diluting Fluid.

Eosin, aqueous 2%	5 ml.
Acetone	5 ml.
Distilled water	90 ml.

The 2% eosin solution is stable and may be kept as a stock solution, but the acetone-eosin mixture must be made up fresh each two weeks and stored in refrigerator.

Procedure: 1. Four-hour test. No food is allowed after 8 P.M. on the night preceding the test. The following morning, under fasting conditions, a direct eosinophil count is performed on oxalated venous blood. (Oxalated blood may be stored for 4 to 6 hours in the refrigerator.) Following the collection of blood for the control count, 25 mg. of ACTH is injected intramuscularly. Four hours later with the patient still fasting the blood is drawn for the second eosinophil count.

2. Two-day test. Prepare the patient as given above. After blood for the control eosinophil count has been taken, 80 units of ACTH gel is

injected intramuscularly. The patient is allowed to eat the usual meals and to have fluids ad lib. Twenty-four hours after the control count has been taken a second injection of 80 mg. of ACTH gel is administered. On the next morning before the patient is given breakfast, that is 48 hours after the control eosinophil count has been determined, a second eosinophil count is performed.

Technique. Draw oxalated blood into the white cell counting pipette to the 0.5 mark. Draw diluting fluid to the 11 mark. Shake gently 50 times. Charge counting chamber. Wait 3 minutes (no longer) to allow the neutrophils to dissolve and for the eosinophils to settle. Count the eosinophils which show up as deep red granular aggregates against a pink background. Use high dry objective and count the eosinophils in the entire ruled area on both sides, that is 18 sq. mm. Using a separate pipette and another sample of blood, repeat the procedure four times, making a total of 72 sq. mm. area counted.

$$\frac{\text{Total No. in 72 sq. mm.}}{72} \times 10 \times 20 = \text{No. eosinophils/cu. mm.}$$

Special counting chambers for eosinophil counting are available which have a larger surface area and a depth of 0.2 mm. One of these is the Speirs-Levy eosinophil counting slide which has 4 chambers per slide. Each chamber consists of ten 1 x 1 mm. squares which are subdivided into 16 smaller areas. Another recommended chamber is the Fuchs-Rosenthal Double Ruling-Levy chamber which has a 16 sq. mm. area on each side (Fig. 1.18).

Interpretation. The normal eosinophil count is 100 to 300 per cu. mm. Following the administration of corticotropin (ACTH), which causes adrenocortical activation, the eosinophil count is decreased. A decrease in the eosinophil count of 50 per cent or more four hours after 25 mg. ACTH intramuscularly or 75 per cent or more after two days of activation with ACTH gel is indicative of normal adrenocortical function and tends to rule out adrenocortical insufficiency. A drop in eosinophil count of less than 50 per cent in 4 hours or less than 75 per cent in 2 days is suggestive, but not diagnostic, of adrenocortical insufficiency.

Erythrocyte Counting

Principle. The blood is diluted with an isotonic solution and the erythrocytes in a portion of the diluted fluid are counted in a hemocytometer under a microscope.

Diluting Fluids.

1. Hayem's Solution
   Bichloride of mercury, 0.5 gm.
   Sodium chloride, 1.0 gm.
   Sodium sulphate, 5.0 gm.
   Distilled water, 200.0 ml.
2. Physiological salt solution, sterile or freshly made and filtered.

Keep well stoppered and filter frequently, as some cells are deposited each time a pipette is inserted.

Procedure. Using the same technique as for the white cell count, draw the blood up to the 0.5 mark in a clean and dry red cell pipette. Wipe the blood from the tip and quickly draw up the appropriate diluting fluid to the 101 mark engraved above the bulb (dilution 1 to 200), rotating the pipette to produce an even mixture and to prevent entrance of air bubbles. Mix the contents by shaking for 2–3 minutes in a transverse direction.

Discard the first few drops from the pipette. Holding the pipette in an inclined position, quickly charge the counting chamber, avoiding overrunning into the moat.

Allow 1–2 minutes for the cells to settle. Using the low power objective, locate the central square millimeter, which is divided into 25 squares bounded by double or triple lines, each one of which is subdivided into 16 smallest squares.

For counting, use the high power objective, adjusting the light so that the red cells and marking lines are clearly defined. First count the erythrocytes in the upper left hand medium sized square, using the system of meander as shown in Fig. 1.29. Cells touching the upper and left boundaries are counted and those touching the right and lower boundaries are ignored. Record the number of cells in the first square. Then proceed to count, in succession, the cells in the upper right corner square, the lower right (Fig. 1.30 EFGH), the lower left and the square in the middle of the field, making a total of 5 medium-sized squares or $\frac{1}{5}$ of the total field. Five diagonally adjacent squares may be counted if preferred.

Some clinicians prefer to count a horizontal row of 20 consecutive smallest squares in each of 4 of the 5 medium sized blocks, making the usual total field of 80 smallest squares. When a mechanical stage is available this method is more rapid than the system of meander and yields comparable results.

Calculation. Since the cells in $\frac{1}{5}$ of a square millimeter are counted,

the dilution is 1:200, and the depth of the counting chamber is $\frac{1}{10}$ mm.; the number of erythrocytes per cu. mm. may be calculated by the following formula:

Erythrocytes in 5 medium-sized squares × 5 × 200 × 10 = number of erythrocytes per cu. mm.

or

Erythrocytes in 5 medium-sized squares × 10,000 = number of erythrocytes per cu. mm.

Example:

Square No	Total Erythrocytes
1	95
2	100
3	105
4	105
5	95
Total	500 × 10,000 (or add 0000) = 5,000,000 erythrocytes per cu. mm.

Normal Values. The erythrocyte count for men varies from 5,000,000 per cubic millimeter to 6,000,000; for women, 4,500,000 to 5,500,000. The average normal value is considered as 5,000,000 per cubic millimeter.

The error inherent in the red cell count is approximately ± 10 per cent when the test is performed by the most skilled and painstaking technicians using standardized equipment. As red cell counts are performed by the average hospital technician, working hurriedly and under pressure to get out reports, the possible errors are approximately ± 20 per cent. Thus if the true red cell count is 5,000,000 per cu. mm. one should expect a range of reported variations to be 4,000,000 to 6,000,000.

Because red cell counts are time consuming and are the least accurate of procedures to detect anemia, they should not be included in routine or screening procedures.

Photoelectric Determination of Erythrocyte Count

In those laboratories in which the authorities insist on performing routine erythrocyte counts as screening tests for anemia, instead of the more accurate and less time-consuming hematocrit or hemoglobin determinations, it is recommended that photoelectric methods be used.

The photoelectric red cell count is more accurate and dependable than the routine counting chamber methods provided the red cells are of average normal size, shape and hemoglobin content, but is not reliable when there is severe anemia.

Photoelectric methods do not eliminate the errors due to faulty collection of blood, improper mixing of oxalated blood and errors in the calibration of pipettes and tubes.

The reader is referred to the excellent articles by L. L. Blum for a discussion of basic principles, technic, the preparation of calibration curves and clinical applications (Am. J. Clin. Path. 15: 85–93, 1945; and 16: 53–57, 1946).

Thrombocyte (Platelet) Counting

Principle. The blood is diluted with a fluid containing a preservative, an anticoagulant, and a stain to aid in visualization. Counts are made with standard blood equipment.

Diluting Fluid. (Rees-Ecker)

Sodium citrate	3.8 gm.
Formaldehyde (40% solution)	0.2 ml.
Brilliant cresyl blue	0.1 gm.
Distilled water	100.0 ml.

Filter final solution, centrifuge to remove particulate matter and store in refrigerator in glass stoppered bottles.

Procedure. Filter a small portion of the diluting fluid into a clean dry test tube. This is an essential step, for precipitates, fungi, cell debris and artefacts are difficult to distinguish from thrombocytes.

Moisten the inside of the red cell pipette with the diluting fluid by aspirating fluid into the bulb and then expelling the excess fluid. Freely flowing blood from a skin puncture wound or oxlated venous blood is then aspirated to the 0.5 mark, followed by Rees-Ecker fluid to the 101 mark (dilution 1 to 200). The pipette is shaken for several minutes, the first several drops discarded and the counting chamber charged. After waiting approximately 10 minutes for the thrombocytes to settle in the counting chamber, the entire central ruled area (400 smallest squares) is counted. The thrombocytes appear among the red cells and occasional white cells as refractive, lilac-tinted objects 1 to 3 microns in diameter. When thrombocytes are grouped the individual units of the group are counted.

Calculation. The number of thrombocytes counted in the central ruled area (1 square mm.) is multiplied by 2,000 to give the number per cu. mm.

Normal Values. The normal number of thrombocytes with the above method varies from 200,000 to 500,000 per cu. mm. The error of the

method is approximately (±)10 per cent. For further discussion of the thrombocyte see Chapter IV.

Errors in Counting Methods

An enumeration of some of the more common sources of technical error includes:

1. Inaccurate calibration of pipettes and counting chambers
2. Defects in coverslips
3. Improper cleaning of glassware
4. Chipped pipettes
5. Inaccurate drawing of blood and diluting fluid to the exact mark
6. Failure to wipe excess blood from tip
7. Diluting fluid contaminated by cells, fungi or artefacts
8. Inadequate or improper shaking
9. Failure to discard diluting fluid from capillary before charging chamber
10. Overrunning of fluid into the moat
11. Drying of fluid in counting chamber
12. Errors in counting
13. Errors in calculation

When oxalated blood is used for cell counting, the counts should be made within two hours after the blood is collected for red cells become hemolyzed and degenerative changes occur in white cells and thrombocytes. Other errors involved in the use of oxalated blood are partial clotting due to inadequate mixture of the blood and anticoagulants and failure to thoroughly mix the sedimented blood cells before the blood is aspirated into the pipette.

Because the number of circulating cells may be significantly altered by exertion, emotional factors, meals and extreme temperature changes, the most accurate blood counts are those done under fairly basal conditions. When serial studies are being made of changes in cellular elements, it is advantageous to make the test at approximately the same time each day.

THE BLOOD SMEAR

The examination of the blood smear is the most informative of any single laboratory procedure. Certain blood diseases such as leukemia, infectious mononucleosis, sickle cell anemia or pernicious anemia can often be diagnosed by the blood smear alone. In numerous diseases the diagnosis can be confirmed or ruled out by the cytological findings. The

examination of the blood film is the method of choice in the diagnosis of parasites of the blood. Smear examination is often of aid in determining whether further procedures are indicated and in checking the accuracy of other laboratory tests. It is of particular value to the general practitioner in making the diagnosis of acute surgical conditions and in guiding therapy. Because the practitioner often collects blood himself, it is essential that satisfactory smears be made, for no amount of skill in staining or diagnostic acumen in the analysis of the cellular pattern will compensate for an improperly made film.

New and specially cleaned slides, wrapped in packets of four, with an accompanying laboratory request slip are recommended for the physician's medicine case. Dirty slides left loose in the doctor's bag and previously used slides with chipped ends and scratched surfaces are not satisfactory for hematological studies.

Coverslip preparations are superior to slide preparations, for the white cells are better distributed, there are more white cells per microscopic field in areas where the cells are thin enough for proper study, there are more suitable fields for study and there is better cell definition. The disadvantages of coverslip preparations are that they are difficult to clean and dry, to transport, to stain, to label and to mount. The use of coverslips is not practical for routine blood smears or for the average practitioner, but they should be used when special studies are being made in laboratories where facilities are available.

If slide preparations are properly made, the same information can be obtained as is possible with coverslip preparations. The disadvantages of slides are that the white cells tend to accumulate at the margins and the ends, where they are piled up, distorted and poorly defined. The larger cells (granulocytes and monocytes) tend to be pushed out to the margins, leaving a disproportionate number of lymphocytes in the middle fields.

Cleaning of Slides and Coverslips

New slides should be washed with soap and warm water, rinsed thoroughly, dried with a clean, lint-free cloth and wrapped in paper or stored in covered boxes. Bon Ami or other types of cleaning substances may be used. Care must be taken that the fingers do not touch the surfaces or ends of the slides, for the film of grease that is left wherever the finger touches interferes with proper spreading of the blood.

Used slides should be left in water until they can be cleaned. This is best done by boiling them in a soda bicarbonate solution or solution of

scouring powder and scrubbing thoroughly. For more thorough cleaning, the slides can be placed individually in cleaning solution made up of equal parts of concentrated sulfuric acid and saturated aqueous potassium dichromate (4.9 gm. per 100 ml. water). After standing 4 hours or longer in the cleaning solution, they should be rinsed in running tap water and dried.

New coverslips should be individually rinsed with warm water and placed immediately in some detergent solution, following which they are rinsed with tap water, distilled water and 70 per cent alcohol. They are placed in clean 95 per cent ethyl alcohol, in which they are stored until dried. Rubber gloves should be worn while cleaning coverslips in order to avoid getting grease on the surfaces. Washed cotton, silk or linen should be used for drying, but surgical gauze may be used. After drying, the coverslips should be placed in covered containers. The small boxes in which coverslips are packed make ideal containers, for these are of such a size that the coverslips can be removed with the fingers without having to touch the surface. If coverslips are stored in larger containers, they should be taken out with coverslip forceps.

Making Slide Preparations

The skin is punctured, the first drop of blood wiped away and the surface of a clean slide is touched to a small drop of blood. The drop should be placed near one end of the slide (Fig. 1.31). The slide is then turned over and the end away from the drop is held by the thumb and forefinger of the left hand. The other fingers serve as a supporting prop (Fig. 1.32). Another slide is picked up and held loosely between the thumb and forefinger of the right hand (Fig. 1.33). This spreader slide is then placed in front of the drop and pulled back until it touches the drop. As soon as the blood spreads by capillary attraction almost to the edge of the slide, the spreader is pushed forward at an angle of approximately 30°, using a rapid, even motion (Fig. 1.34). The weight of the slide is the only pressure applied. The thickness of the spread is controlled by the size of the drop and the angle at which the spreader slide is held. The greater the angle, the thicker and shorter is the film. Several good preparations should be made. Allow the slide to dry in the air. After drying, write the name of the patient and the date in the thick end of the blood film, using a pencil or the end of another slide. Place the slides in a slide box or wrap them separately in a laboratory request slip.

Some workers prefer to use a table top as a support for making the blood smear, but doctors should learn the technique in which the hands

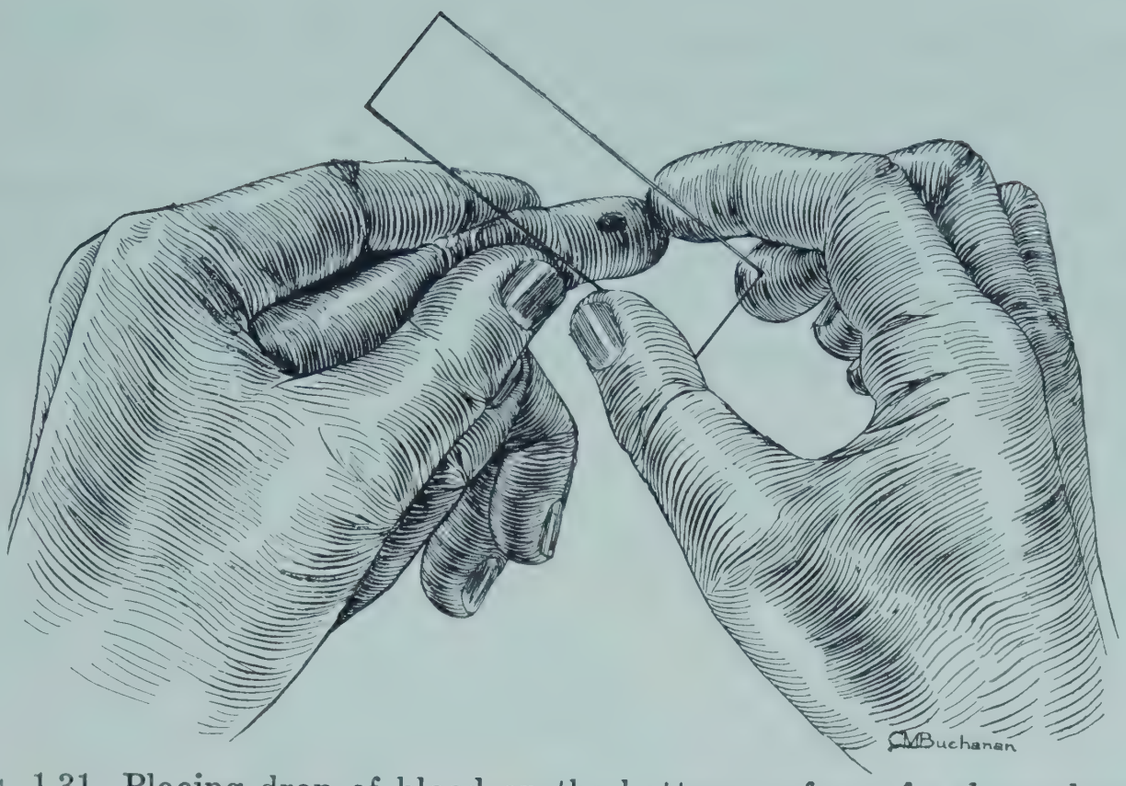


FIG. 1.31. Placing drop of blood on the bottom surface of a clean glass slide The drop should be small and placed near the end of the slide.

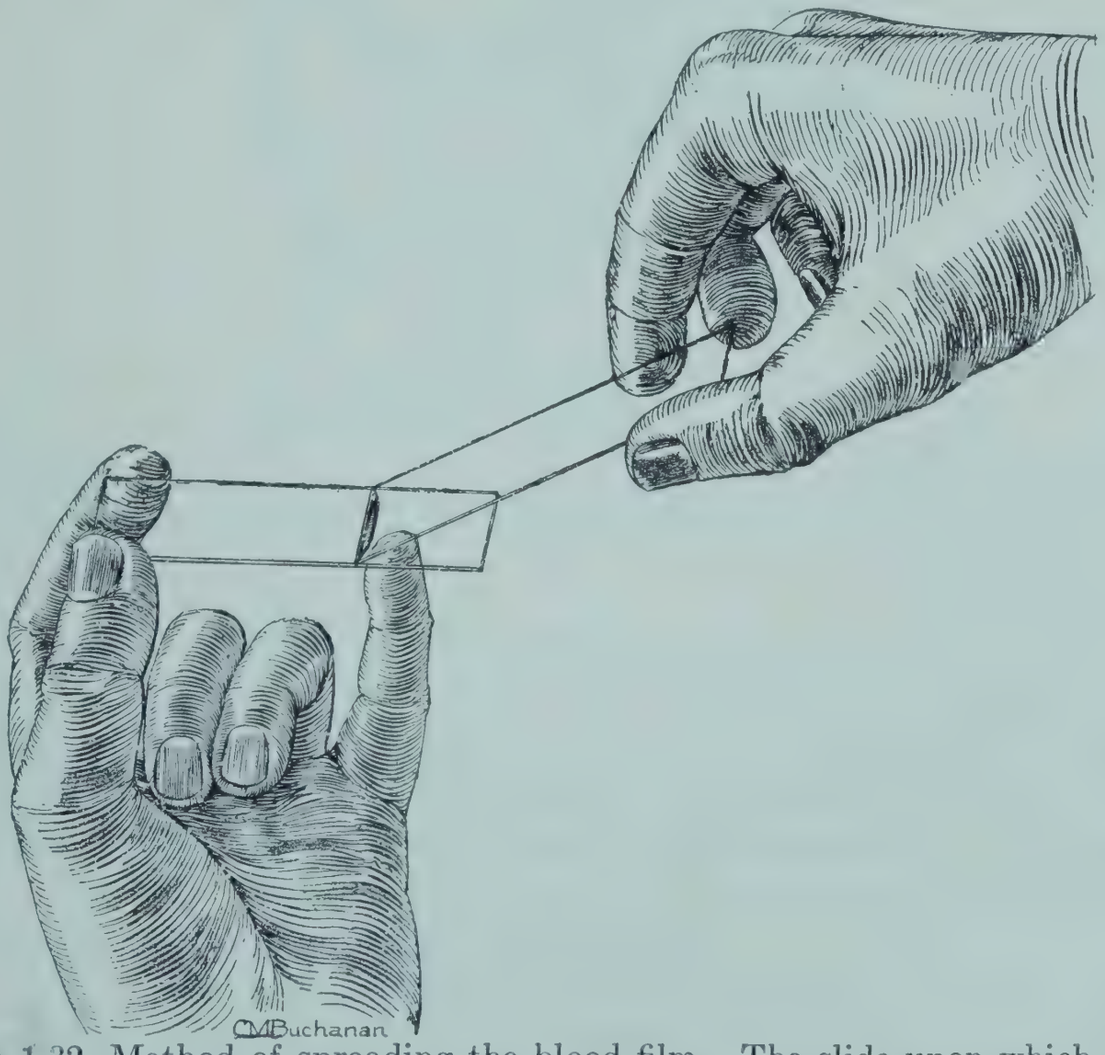


FIG. 1.32. Method of spreading the blood film. The slide upon which is the drop of blood is held in the left hand as shown or is placed on a flat surface. Holding the spreader slide in the right hand as illustrated the edge is backed into the blood drop which spreads across the slide. Then holding the spreader slide at about thirty degrees it is pushed forward rapidly and smoothly.

are used, for suitable stands and adequate light are frequently not available in the home, office or hospital.

A good slide preparation should be thick at one end and thin at the other end. This thin area, in which the red cells are well separated, should be at least 2 cm. long (Fig. 1.36). The film should occupy the central portion of the slide and should be margin free on all sides. Long streamers on the ends of smears are caused by dirty or chipped spreaders.

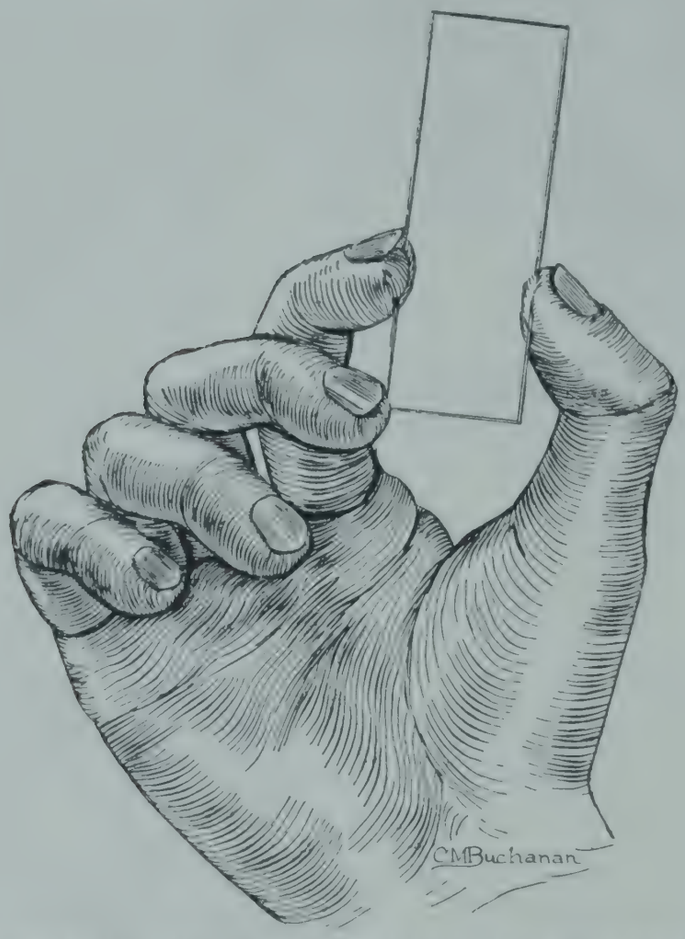


FIG. 1.33. Position of the clean slide that is to be used as the spreader slide in making a blood film. Note that it is held by the edges between thumb and forefinger of the right hand.

Holes in the film are indicative of grease. Streaks are caused by fibrin or dust particles (Fig. 1.35).

Avoid placing slides near a radiator, sink or steam bath, for moisture causes the red cells to become hemolyzed. The blood film should also be protected from flies and cockroaches, which is best done by turning the slide over on a piece of paper or using a covered slide box.

Making Coverslip Preparations

Square coverglasses, 22 x 22 mm. No. 1 or better grades of No. 2, are used. With forceps, remove coverslips from the container and stand them upright in slots cut in a paper box. Puncture the skin and dry

the surface. Hold a coverglass by adjacent corners between the thumb and forefinger of the left hand. With the right hand express a small drop of blood from the puncture wound. Touch the surface of a second coverslip to the drop. The drop should be small, so that on spreading, it will not reach all edges of the glass square. Place the second slip on the first diagonally. If the coverslips are clean, the blood will immediately spread by capillary action. Grasping the top cover-glass by ad-

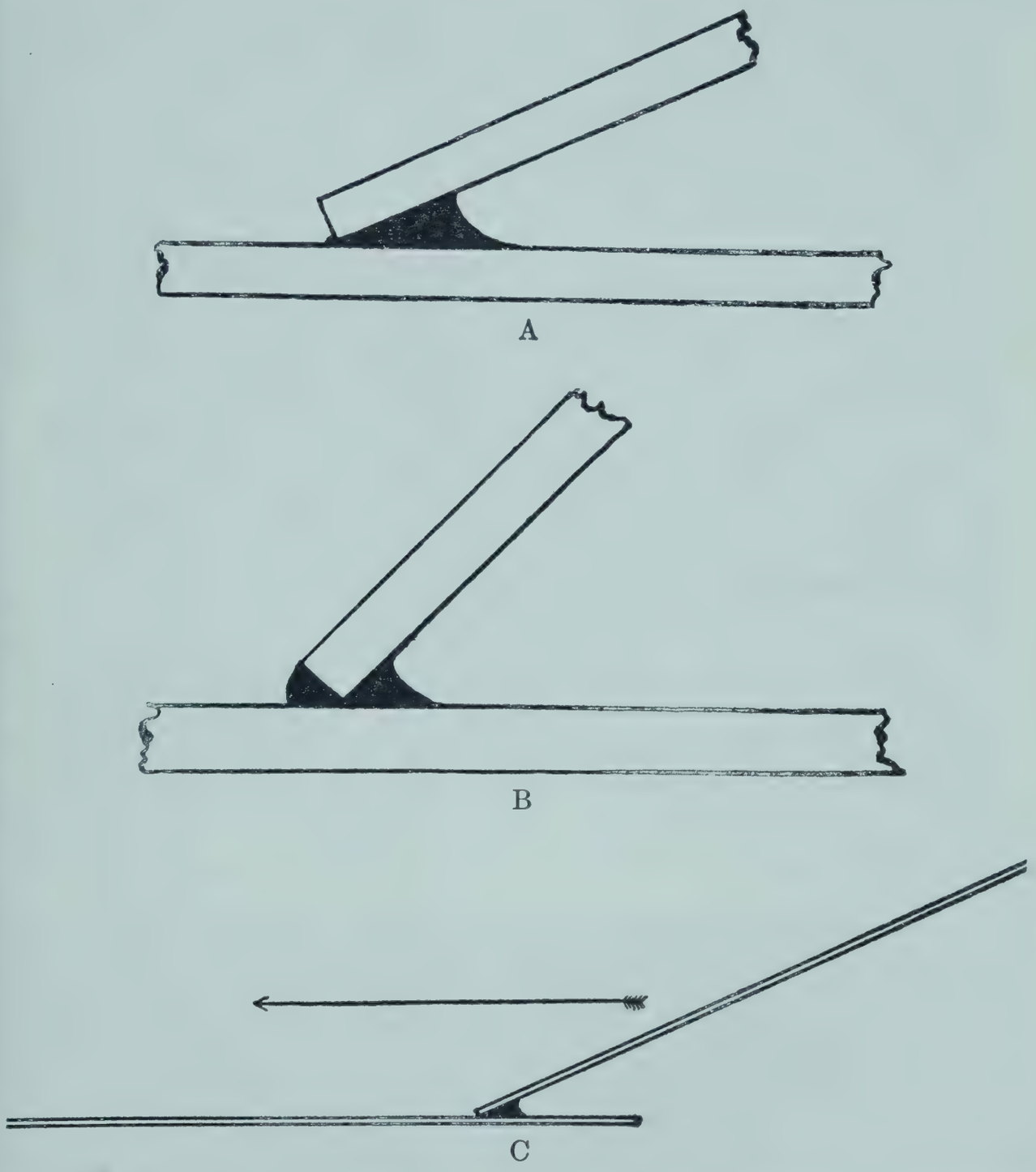


FIG. 1.34. A. Showing how the blood collects behind the edge of the spreader slide when the angle between the two slides is small. B. Showing how blood collects in front of the edge of the spreader slide when the angle is too great. C. Showing the direction in which the slide is moved to make the blood film.

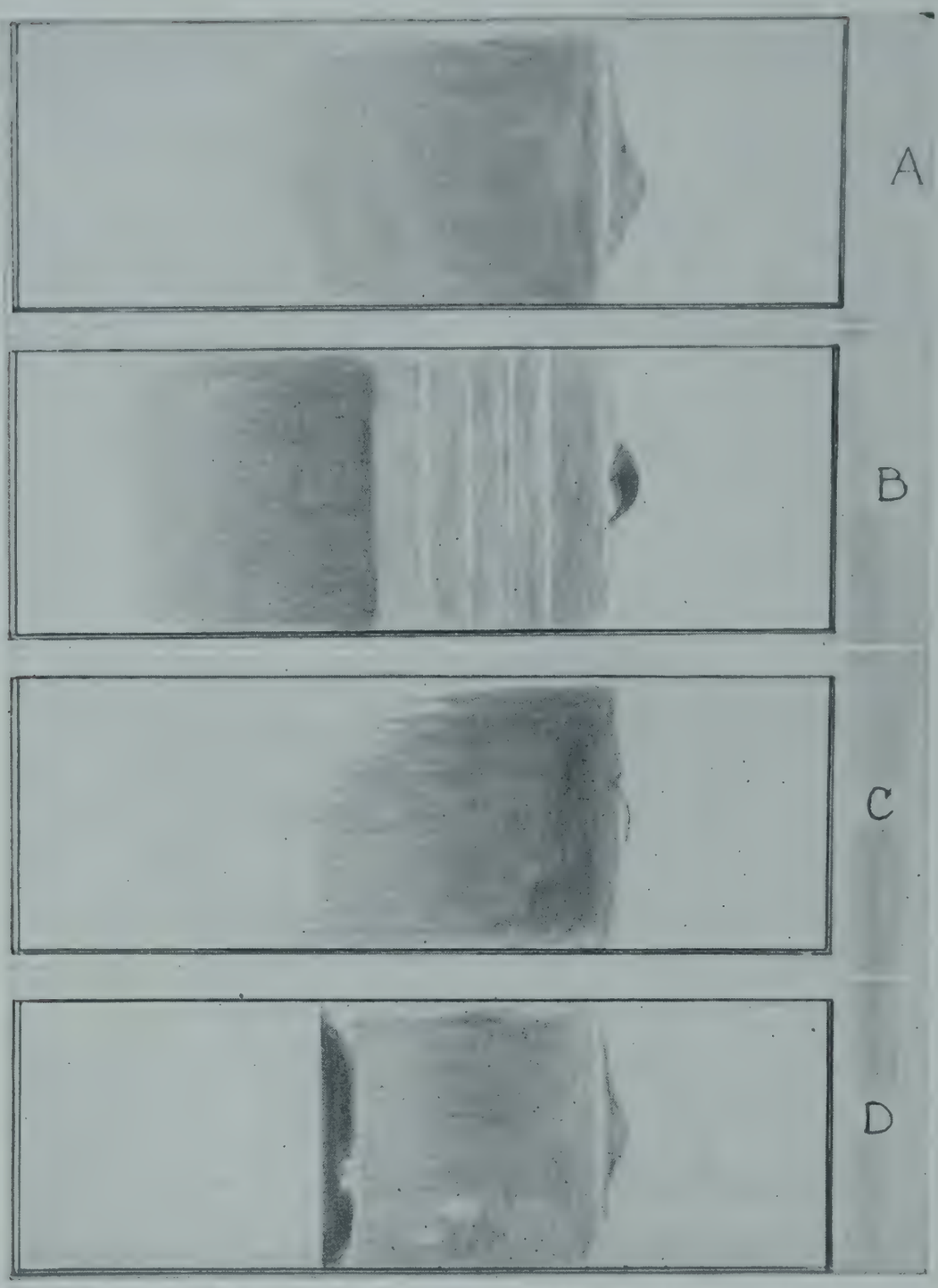


FIG. 1.35. Improperly made blood smears. A. Too short, not margin free. B. Irregular pressure. C. Too short and thick, jagged ends due to irregular spreader. D. Thick area at end due to spreader hitting supporting finger.

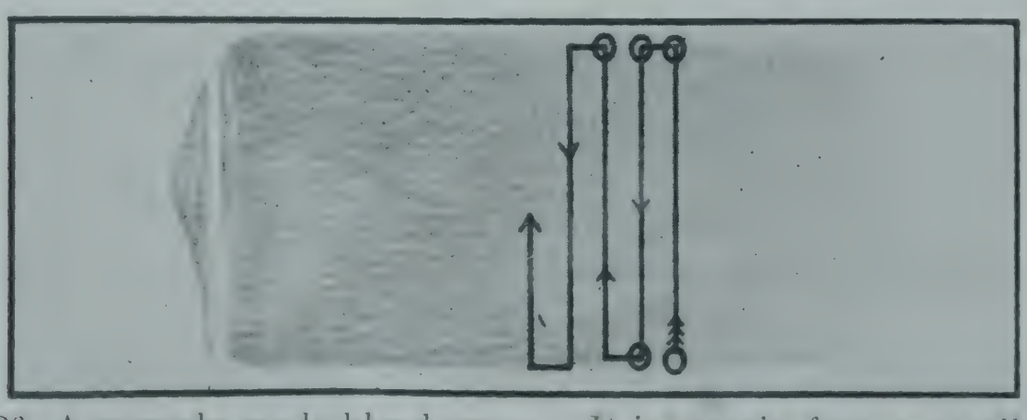


FIG. 1.36. A properly made blood smear. It is margin free, centrally placed and has an adequate thin area. In counting use system of crossing as in diagram.

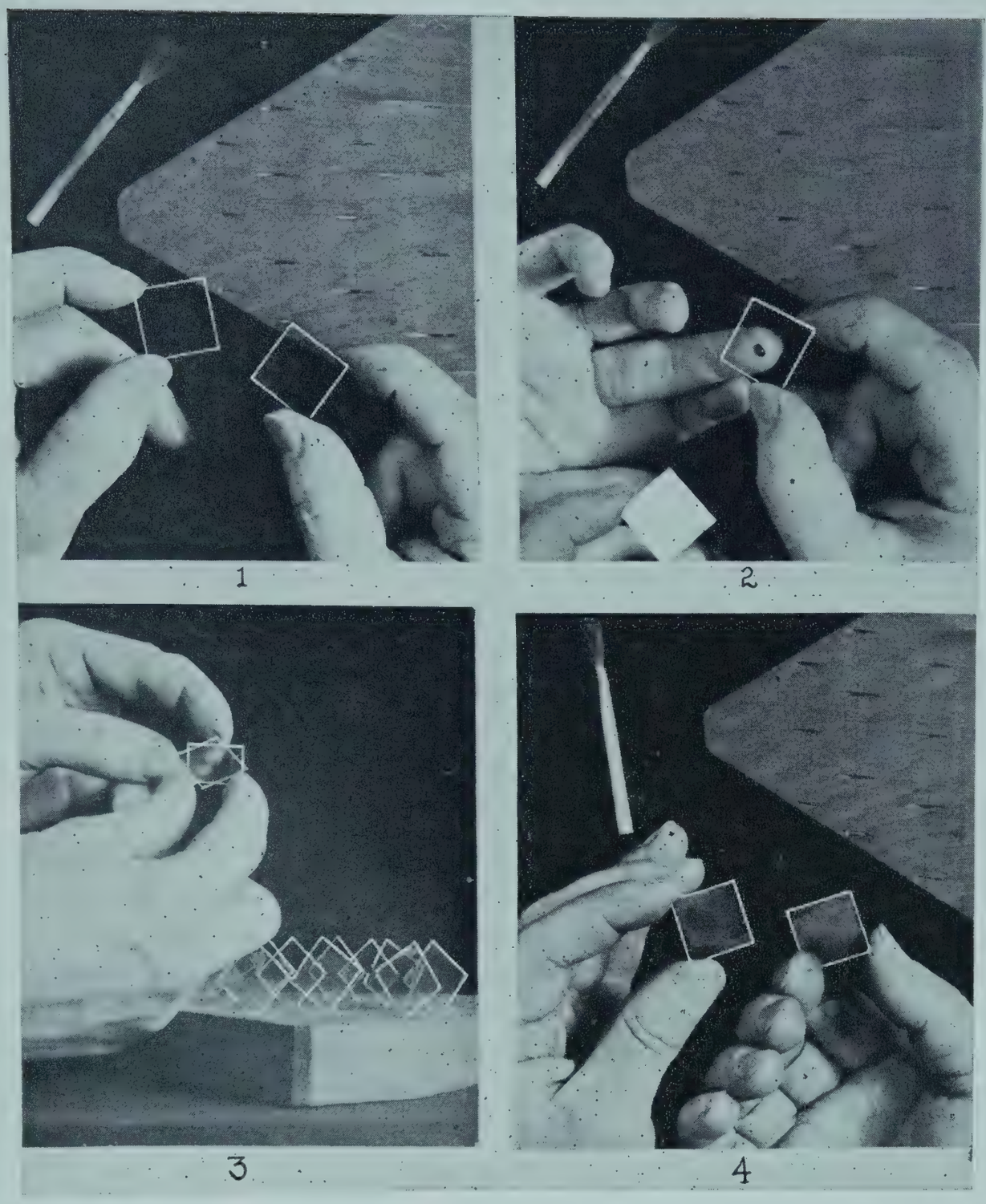


FIG. 1.37. The preparation of blood films by the cover glass method. 1, a cover glass ($\frac{7}{8}$ inch square No. 2) is grasped at the adjacent corners with the thumb and forefinger of each hand; 2, the drop of blood is touched with the cover glass held in the right hand; 3, the cover glass carrying the drop of blood is quickly placed on the cover glass held in the left hand; 4, cover glasses are then drawn apart with a sliding motion, care being taken to keep them parallel. The films are allowed to dry in air and are then ready for staining. The drop of blood must be globoid on the finger tip and just large enough to cover the cover glass when properly spread. (From Haden, *Clinical Laboratory Methods*.)

jacent corners and using a smooth outward motion, pull the slips apart (Fig. 1.3[7]). In making the pull, it is well to have the wrist stiff and to use the arm and shoulder muscles. Allow the smear to dry in the air. If the preparations are made in the laboratory, they can be placed on a

sheet of paper on which the patient's name and the date are written. If taken at the bedside, the coverslips can be placed in a small pillbox containing a label or placed back in the slots of the cardboard box.

Good coverslip preparations should have no holes or streaks and should have numerous consecutive fields in which the red cells are well separated and the white cells thinly spread.

Staining

Dried blood films protected from dust, insects and moisture remain in good condition for several days, but gradually undergo degenerative changes. For the best detail, the films should be stained soon after they dry.

Numerous stains and staining techniques are used, but the most commonly used stains are made from basic and acid aniline dyes, which bring out in contrasting colors the red and blue staining elements. The most widely used stains contain methylene blue or one of its derivatives and eosin and are known as "Romanowsky" stains, the best known modifications of which are Wright's and Giemsa stains. Stains certified by the Commission on Standardization of Biological Stains are recommended.

Structures which stain with the red eosin dye are said to be eosinophilic, acidophilic or oxyphilic, whereas those that stain with the blue dye are said to be basophilic.

A suitable rack for staining coverslips is made by nailing small corks on a board. A suitable rack for slides or coverslips is best made by passing straight glass rods through two two-hole rubber stoppers and laying them across a staining pan. Staining is best done on the drainboard of a sink, or near a sink, so that one may easily dispose of the wash water. Stock stains should be kept in tightly stoppered bottles and dispensed in small dropping bottles. The stain bottles should not be left near strong acids or alkalies and care must be taken that water or other fluids do not come in contact with the glassware. The buffered water and distilled water used for diluting and washing are best stored in large jars with a siphon type of U-tub and stopcock attached and placed on a shelf above the staining rack.

Wright's Stain. This stain can be purchased from laboratory supply houses in a liquid form ready for use or it can be purchased as a powder (National Aniline and Chemical Co.; Coleman and Bell). The powder is dissolved in acetone-free methyl alcohol (Merck's reagent or Mallinckrodt absolute).

In order to make up 500 ml. of stain, weigh out 0.9 gm. of dried powder

and place in a clean mortar. Add a little acetone-free methyl alcohol and grind to facilitate dissolution. Pour the dissolved dye into a bottle having a tightly fitting glass stopper. Add more methyl alcohol, grind, pour off and repeat the process until all of the alcohol is used. Shake the bottle of stain at least once each day for five days. Allow the precipitate to settle and be careful that the stain is not agitated when the supernatant fluid is poured off into dropping bottles for use. Filtration through a fine filter paper can be employed, but this step in the procedure should be avoided unless haste is essential.

Distilled water or buffered water are used for diluting the stain and for washing. The pH of the water is critical and tap water is seldom satisfactory. The following phosphate buffer mixture is recommended: 43.5 ml. of M/15 Na_2HPO_4 (anhydrous, 9.5 gm. per L.) and 56.5 ml. of M/15 KM_2PO_4 (9.07 gm. per L.) plus 900 ml. of distilled water (pH 6.7).

Certified buffer tablets in vials of 12 with hermetic wax seals can now be purchased (Coleman Instruments, Inc., Maywood, Ill.) One of these tablets dissolved in 100 ml. of distilled water gives the desired pH value, which for the Wright's stain is 6.6 to 6.8.

The pH of the buffer may be checked by the use of simple color standards containing an indicator with a pH range around neutrality, such as bromthymol blue. Hematoxylin, in crystalline form, may be used as the indicator by placing several crystals in the test tube of water to be tested. A lavender-pink color in two minutes is produced if the water is neutral, reddish-purple if alkaline and yellow if acid. The pH of the water can be changed by adding to the stock bottle a 1 per cent aqueous solution of potassium carbonate or 1 per cent hydrochloric acid, a drop at a time, a sample of the solution being tested after each addition.

Place the slide or coverslip in a rack in a perfectly horizontal position and add Wright's stain to completely cover the surface. If too much is used, the stain will run off when the water is added, and, if too little is used, the stain will evaporate, leaving undesirable deposits behind. Do not shake the bottle containing the stain, for this may cause precipitates on the slides. Allow the stain to stand 1 to 2 minutes.

Then add an equal number of drops of buffered water to bring the meniscus to the margin. Leave the mixture on for 4 to 6 minutes. A green metallic sheen is indicative of proper staining. Each batch of stain must be tested for ideal timing. Blood smears containing many cells and bone marrow smears require a longer staining time than do thin preparations or smears with few white cells.

Wash the stain off with distilled water. Do not pour the stain off

before washing, because this will leave a precipitate. Wipe the bottom of the slide to remove dried stain. Place the slides or coverslips endways on filter paper to allow the excess fluid to drain off. Vigorous blowing on the slide or the use of a fan or electric dryer speeds up the drying process and leaves a minimal deposit.

A good Wright's stain is pink and characterized by erythrocytes which are light buff color, nuclei which are sharply stained blue with contrasting light cytoplasm, eosinophilic granules which are bright red and neutrophilic granules, pink or light purple. There must be no stain or precipitate between the cells.

It is a good idea to examine under low power to see if the preparation is properly stained before mounting or adding mineral or cedar oil. If the nuclei are too lightly stained, place the slides back on the rack and restain. If precipitates are present or if the background is bluish, add Wright's stain, allow to stand a few seconds and wash. Methyl alcohol may be used as a solvent, but must be used cautiously, as it will rapidly take the stain out of cells. Filtering of the stain will eliminate some of the precipitates.

If the stain is too dark and too blue, this may be partially corrected by shortening the staining time, using less stain and more diluting fluid or making the buffered water slightly more acid. If the stain is too red, or the neutrophilic granules invisible, the buffered water can be made more alkaline and the staining time increased.

Giemsa Stain. Giemsa stain is especially valuable for staining large numbers of slides simultaneously, for the laking and staining of thick films and for the diagnosis of parasitic diseases. It is not as satisfactory as Wright's stain for routine hematological procedures, because the staining time is longer, preliminary fixation is required and the neutrophilic granular detail is not as good.

The stock alcoholic Giemsa solution is diluted 1 to 10 for the staining of ordinary blood smears, but for the laking of erythrocytes and the staining of thick drops, dilutions up to 1 part of stain in 50 of buffer may be used. (See under Malaria for discussion of procedure Chapter 14.)

The buffered water used in diluting the Giemsa stain should be neutral

pH	Na_2HPO_4 (9.5 gm. per L.) (anhydrous)	$NaH_2PO_4 \cdot H_2O$ (9.2 gm. per L.) or KH_2PO_4 (9.07 gm. per L.)	Distilled Water
7.0	61.1 ml.	38.9 ml.	900 ml.
7.2	72.0 ml.	28.0 ml.	900 ml.

or slightly alkaline. A satisfactory preparation according to Wilcox is made by preparing stock solutions of phosphates and adding measured amounts of these to distilled water to give the desired pH.

Since the Giemsa solution is diluted before use, it is necessary to fix the blood smear before it is stained. This is done by flooding the blood film with methyl alcohol or by dipping the slide in a jar or dish of alcohol for 1 to 5 minutes. The alcohol is drained off, allowed to dry and the dilute Giemsa solution added, or the slide is immersed. For the ordinary blood smear, 15 to 30 minutes are required; for bone marrows, exudates and thick drop preparations, 30 minutes or longer, and for spirochetes and certain protozoa, 60 minutes or more.

After staining, wash the slide with distilled water. Dry and examine.

Wright's stain can be combined with Giemsa by using dilute Giemsa solution as a diluting fluid, or by placing the slide, after staining with Wright's, in a dilute Giemsa solution for 10–20 minutes. Wilcox describes the preparation of a Wright-Giemsa stain as follows: Dissolve 2 gm. Giemsa powder (National Aniline Chemical Co., Inc., New York) in 100 ml. glycerine (C.P. from a freshly opened bottle). This may be done by heating in a water bath at 55° to 60°C. for 2 hours and mixing well at intervals with a glass stirring rod, avoiding absorption of moisture. To this mixture add 100 ml. Wright's stain solution (aged solution of 2 gm. powder to 1,000 ml. methyl alcohol). Let stand over night and then add an additional 800 ml. of aged Wright's solution. Filter and use.

The combination stain is often useful in salvaging smears in which cells are feebly stained and in staining more deeply objects that are not readily stained by Wright's alone.

Mounting and Labeling

Coverslips, after being stained, are mounted on slides by placing a drop of mounting material on the stained surface and placing it in the middle or toward the end of a clean slide.

If the slide and coverglass are too thick to enable use of oil immersion examination, the coverslip can be mounted with the blood film uppermost. Slide preparations do not require mounting unless they are to be preserved for future studies or for repeated examination. "Permount," a synthetic resin, is recommended.

Slide preparations can be identified by writing directly on the blood film at the time the preparation is made, but for filing, it is preferable to place a gummed label at the thick end. The label should contain the name of the patient, date and other useful information.

Examination

Many prefer to examine the smear under high dry magnification. For thoroughly trained workers and for the routine examinations of blood smears, examination using the high dry objective is recommended, as it saves much time. For less experienced examiners and for the study of pathological blood cells, the oil immersion objective and 6X or 10X eyepieces are used.

It is advisable that the examination start at the thin end and that a systematic meander be followed (Fig. 1.36). In this way, reduplication of the field is avoided. A mechanical stage greatly aids in orderly examination of the field. Fields should be examined in which the red cells are well-separated and the white cells thinly spread, and fields avoided in which the red cells are stacked up or are in rouleaux and the white cells spherical. For slide preparations, it is necessary that the margins be included in the count.

In recording the different cells found, the types of cells are first written on scratch paper. Sheets of paper on which the cell types are stamped or mimeographed save much time. As the individual cells are paraded past the field of vision, they are individually classified, a single stroke being used for each cell and a cross stroke or tally being used for each fifth cell. A one-unit hand tally counter may be used to record the total number of cells counted, or counters with multiple units, such as the 9-unit lab. tabulator or the Marble Percentage Calculator, may be used for the individual cell types and the total (Fig. 1.38). When using the single unit tally counter, a good procedure is to make tally strokes opposite all cells except the segmented neutrophils and to arrive at the



FIG. 1.38. Blood cell counter for use in differential counts. (*Courtesy of The Denominator Co., Inc.*)

number of these cells by subtracting the number of all other cells from the total.

In practically every blood smear, there are a few degenerated, distorted and atypical cells which cannot be classified accurately. If such cells are few in number, it is sufficient to simply classify them as "atypical." If one suspects that a cell is a monocyte, for example, but is not sure, he can record this cell with a question mark instead of a tally mark. If the cell is early or abnormal, or has peculiar granules or vacuoles, symbols can be used to indicate these features so that at the end of the differential the operator can state, not only that these abnormalities exist, but be able to estimate the percentage showing such features. Often it is impossible to tell exactly what type of cell is present, but the characteristics are sufficient to say that it is one of two possibilities. In such instances, it is convenient to start a new column, such as "? monocyte—? lymphocyte" or "? monocyte—? metamyelocyte." In pathological bloods in which there are numerous abnormal cells of a given type, it is convenient to divide these cells into early, intermediate and mature cells.

Often the examiner, on first examining the smear, has many cells that he cannot classify; but after examining several hundred cells, and listing the typical cells, he can bridge the gap between the abnormal and the normal cells and make a diagnosis of abnormal cells by association with the known. One has to take into consideration the stain, the presence of precipitates and the thickness of the spread, for alterations in technique may create differences in the morphology of cells. The diagnosis of cells by the other cells in the same preparation (diagnosis by association) is extremely important. Thus, if there are quite a few cells which could be metamyelocytes or monocytes, but there is no increase in the non-segmented neutrophils and there is an increase in typical monocytes, the questionable cells are monocytes. On the other hand, if there is a definite increase in neutrophils with an increase in non-segmented neutrophils and few typical monocytes, the unknown cells are classified as myelocytes or metamyelocytes.

The source of the smear, also, is used to make decisions. In a spread from peripheral blood of a patient with myelocytic leukemia or from a bone marrow, an atypical granular cell would be placed in the neutrophil column rather than with the monocytes; a cell which is borderline between a lymphocyte and a nucleated cell is called a lymphocyte in peripheral blood and a nucleated red cell in bone marrow. One should be hesitant about calling any cell a blast unless there are unmistakable

TABLE 1.1

Examination of Peripheral Blood

Name..... Date.....

.....

Age..... M F W C Referred by.....

Red Blood Cells

Thrombocytes

White Blood Cells Number counted.....

Normal		Per cent
%	Myeloblast	
	Progranulocyte	
	N. Myelocyte	
0–1	N. Metamyelocyte	
2–10	N. Band	
50–70	N. Segmented	
1–4	Eosinophil	
0–1	Basophil	
	Lymphoblast	
	Prolymphocyte	
20–40	Lymphocyte	
1–6	Monocyte	
	Plasmocyte	
	Atypical cell	

Disintegrated cells per 100 intact WBC

SUMMARY OF ABNORMALITIES

INTERPRETATION

Examined by.....

morphological characteristics and supporting evidence. In case of doubt, it is better to report such cells as "atypical early."

The minimal number of white cells counted in arriving at a differential is 100, but, if there are abnormal cells, or a blood dyscrasia is suspected, 200 or more cells should be counted.

While the white cell differential is being determined, the red cells and thrombocytes should be examined and a search made for parasites, stippling, diffuse basophilia and other red cell abnormalities. If nucleated red cells are seen, they should be classified according to the stage of development and enumerated. In peripheral blood the nucleated red cells are usually reported as the number per 100 white blood cells.

Smudges should be tallied while doing the differential count and reported in terms of number per 100 non-smudged white cells.

A suggested form for the reporting of the findings in a blood smear is given in Table 1.1.

TECHNIQUE FOR DEMONSTRATING "L.E." CELLS IN BLOOD

Draw 10 ml. of peripheral blood in a serological tube and allow it to clot. After 2 hours at room temperature, rim the clot if necessary and remove the serum. Remove the cells from the clot by mashing through a piece of wire gauze placed over a Petri dish or other container. The end of a test tube may be used. A special sieve and pestle (American Hospital Supply Company #68401) for this purpose can be purchased. Fill a Wintrobe sedimentation tube with the blood expressed. Centrifuge at 2,000 r.p.m. for 5 minutes. Withdraw the serum down to the layer of leukocytes. Make smears from the buffy coat of white cells. Stain with Wright's stain and examine.

Look for polymorphonuclear leukocytes containing velvety homogeneous round or lobular structures, which stain various shades of purple. Also search for rosettes of polymorphonuclear leukocytes clustered around structureless red-staining material. See Chapter 2.

SPECIAL STAINING PROCEDURES

Supravital Stains

Supravital stains are relatively non-toxic dyes which act upon cells in their living state, causing certain structures to be more readily visualized and allowing studies of motility and phagocytosis. Weak solutions of dyes such as neutral red, pinacyanol and janus green in alcohol are flooded onto a slide, the excess drained off and the alcohol allowed to dry, thus leaving a thin film of dye. A small drop of blood on a

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coverslip is placed in contact with the dye and the margins of the moist preparation rimmed with Vaseline.

Neutral red is an indicator which reacts with the specific granules of the leukocytes and appears in the vacuoles of the phagocytic cells, giving varying shades of red and yellow, depending on the chemical properties of the cell structures. Janus green and pinacyanol stain mitochrondria.

Study of cells by supravital techniques has added much to our knowledge of cell morphology, motility and function. The procedure is used in some hematology laboratories for differential counts, but is time consuming, difficult, and too expensive for general use. For details of technique, characteristics of the various cells and a discussion of the advantages and disadvantages of methods, and a more complete bibliography, the reader is referred to articles by Hall, Hetherington, Schwind, Tompkins and Cunningham.

Peroxidase Stain (Sato and Sekiya's Method)

Principle. The peroxidase stain depends on the presence of an oxidizing enzyme in the granulocytes, which in the presence of peroxide and copper sulfate acts as a catalyst in the oxidation of benzidine, producing a blue-black compound in the granules.

Solutions.

A. 0.5 per cent aqueous copper sulfate.

B. Saturated aqueous solution of benzidine.

Rub up in a mortar 0.2 gm. benzidine with a few drops distilled water. Add 200 ml. distilled water and filter. To the filtrate add 4 drops of 3 per cent hydrogen peroxide.

C. 1 per cent aqueous solution safranin.

Procedure. Solution A is applied to a dried blood film for 1 minute. The copper sulfate solution is poured off and Solution B is added and allowed to remain for 2 minutes. The film is then washed with water and counterstained with Solution C for 2 minutes. After drying, the smear is examined and the staining reaction of 200 nucleated cells catalogued in three categories: 1) Heavy granules 2) Few granules and 3) No granules.

A good stain is characterized by heavy blue black granules in cells which have polymorphonuclear nuclei. Eosinophilic and neutrophilic granules give a strongly positive reaction. Lymphocytes, plasmocytes and nucleated red cells are stained red but react negatively. Monocytes give a variable reaction, but as a rule have few specific granules. Basophilic granules as a class do not react. The most primitive cells of all

series give a negative reaction, but myelocytic cells may reveal definite positivity in cells that do not reveal specific granules in the Wright's stain. The principal use of the peroxidase stain is in differentiating lymphocytic leukemia from myelocytic leukemia of a stem cell type, but the stain is of little value in differentiating early monocytes from early myelocytic cells.

If no peroxidase positive cells are found in a given preparation, test the reagents using a normal blood smear, for Solution B may deteriorate on standing. A few drops of fresh hydrogen peroxide will often restore the activity of this reagent.

HEMOBLOBIN ESTIMATION

General

Although the estimation of hemoglobin was one of the first of the hematological methods to be developed, and is one of the most useful and widely used procedures, it is perhaps the most inaccurately performed and reported of all quantitative biochemical tests. This is in part due to the fact that hemoglobin is an unstable compound and is not available as a weighable substance for the purposes of standardization. The indirect methods of standardization such as the iron concentration and oxygen capacity methods require a degree of chemical skill and special equipment which is not available in the average laboratory.

Another reason for inaccuracies in hemoglobin estimation is that the first crude instruments which were developed used arbitrary standards and reported the results in terms of per cent of a so-called "normal value." There has never been an agreement as to which value is most representative of normal. As new instruments and methods were developed, each manufacturer used his own standard, the result being that aliquots of blood on one instrument might read 100 per cent and on another 85 per cent, and neither would reveal the quantity of hemoglobin present. There can be no one value for hemoglobin which is normal for all individuals, since the concentration of hemoglobin varies with age, sex and altitude. The reporting of hemoglobin in per cent belongs to the horse and buggy days and should be relegated to the museum of interesting but useless antiques along with the saddle bag and divining rod.

There is one way to report hemoglobin and that is in terms of absolute value or grams per 100 ml. No clinician should be willing to accept the report of hemoglobin in terms of per cent.

Selection of Methods

There are numerous hemoglobin methods and modifications of methods. The procedure and instrument chosen will vary with the type of work being done, the number of tests to be performed and the personnel and instruments available. The methods recommended for various uses are given in Table 1.2.

Several methods are available which do not require pipettes or diluting fluids. The Dare hemoglobinometer (Fig. 1.39) compares the redness

TABLE 1.2

Hemoglobin Methods Recommended

Use	Method	Instrument	Approximate Error
			%
Standardization	Iron concentration	Photoelectric colorimeter	±1
Hospital, clinic and larger laboratories	Oxyhemoglobin	Photoelectric colorimeter	±2
	or		
	Acid hematin		±5
Office practice, medical student use	Acid hematin	Sahli or Haden-Hausser	±10
Screening procedures, home practice	Color comparison of blood with red color scale	Tallqvist	±20

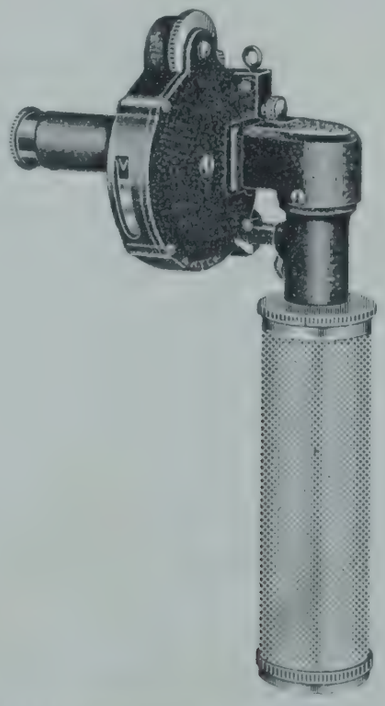


FIG. 1.39 The Dare hemoglobinometer. Equipped with a battery in the handle of the instrument. This instrument can also be obtained so designed to utilize electric current. It involves the principle of matching unaltered red blood against the variations in a red circular scale.

of a drop of blood placed in a capillary chamber with a series of red standards on a revolving disc. The Spencer Hb-Meter utilizes a drop of blood which has been laked with saponin on an applicator stick. The laked blood is placed between two closely approximated glass plates, and the concentration of hemoglobin measured by means of a sliding wedge and a filter which utilizes light in the visible green spectrum in which hemoglobin shows the maximal light absorption. Of the two, the Spencer Hb-Meter is the more accurate. Both instruments are fairly expensive and do not lend themselves readily to the performance of multiple tests, but are useful for a physician who does occasional tests and does not have technical assistance.

In detecting anemia in prospective donors for transfusion, various dilutions of copper sulfate have been used, such as a dilution of copper sulfate with a specific gravity of 1.052 which corresponds to a hemoglobin value of 12.3 grams. A drop of blood from a skin puncture wound is allowed to fall into the copper sulfate solution. If the drop rises promptly it is assumed that the hemoglobin is less than 12.3 grams (Boynton, M. H.: J. Lab. & Clin. Med. 31: 40–44, 1946).

Standardization of Instruments

All hemoglobinometers require standardization even though recently purchased.

The so-called permanent glass standards are not permanent and photoelectric cells do not stay constant. Photoelectric instruments are notoriously subject to mechanical defects. Fluctuations in electrical current may cause significant variation in reading. Hemoglobin readings which are consistently high or low and mean corpuscular values which are not consistent with the appearance of the red cells in the stained smears are indicative of a need for standardization.

One of the most practical methods of standardizing hemoglobinometers is to determine the packed cell volume of a small group of normal individuals and to estimate the hemoglobin concentration on specimens from the same blood samples. The hemoglobin expressed in grams per 100 ml. should be $\frac{1}{3}$ that of the packed cell volume expressed in ml./100. Thus, if a person has an hematocrit of 45 ml. and red cells of average normal size containing a normal amount of hemoglobin, the hemoglobin concentration should be 15 gm./100 ml.; if the hematocrit is 48 the hemoglobin should be 16 gm., etc. Therefore the photometer dial reading corresponding to 1 gm. of hemoglobin is estimated by dividing the photometer dial reading of the blood whose hematocrit has been determined previously by $\frac{1}{3}$ of the hematocrit reading.

For example: photometer dial reading 200, hematocrit 45 ml./100 ml.

$$\frac{45}{3} = 15 \qquad \frac{200}{15} = 13.3$$

photometer dial reading for 1 gm. of hemoglobin equals 13.3 (therefore the reading on a sample containing a hemoglobin concentration of 10 gm./100 ml. would be 133).

The instrument is standardized by determining the average dial reading for 1 gm. of hemoglobin, based upon the hematocrit determinations of a small series of normal individuals. This method is not ideal since it has to be assumed that the persons used for standardization are normal.

The two chemical methods used most frequently for the standardization of hemoglobinometers are the oxygen capacity method of Van Slyke and the iron concentration colorimetric method of Wong. One gram of hemoglobin is equivalent to 1.36 volumes of oxygen. Iron constitutes 0.34 per cent of the hemoglobin molecule.

For details concerning the calibration of hemoglobinometers, the reader is referred to the texts on biochemistry and to the *Symposium on Clinical Hemoglobinometry*, Am. J. Clin. Path. 23: 519–598, 1953.

It is necessary that the pipettes and test tubes used in hemoglobin procedures be calibrated and that no pipettes be used until they have been tested. The most satisfactory procedure for calibrating hemoglobin pipettes is to weigh the mercury which is drawn up to the 20 cu. mm. mark (Stevenson, G. F., Smetters, G. W. and Cooper, J. A. D.: Am. J. Clin. Path. 21: 489–91, May 1951).

Methods

Oxyhemoglobin Method. *Principle.* Red blood cells are laked in 0.04 per cent ammonium hydroxide and shaken with air thereby converting the hemoglobin to oxyhemoglobin. The pigment is measured by means of the photoelectric instrument.

Reagents. 4 ml. of concentrated ammonium hydroxide is added to 1,000 ml. of distilled water.

Equipment. Klett-Summerson photo-colorimeter with green filter of wave length 540 mm.

For blood from skin puncture wound, Sahli hemoglobin pipettes, with graduation at 0.020 ml. mark and Klett-Summerson tubes calibrated at 5 ml.

For oxalated blood, micropipettes with graduation at 0.1 ml. and test tubes or flasks graduated at 25 ml.

Procedure. Place 2 to 3 ml. of 0.04% NH_4OH in a Klett-Summerson tube. Puncture skin. Aspirate freely flowing blood to 0.02 mark. Do not go above line for this will create error. Wipe off excess blood from tip of pipette. Discharge blood into alkaline solution. Rinse pipette three times to obtain all of hemoglobin. Make up to 5-ml. mark with 0.04 per cent NH_4OH solution. Oxygenate by inverting the tube several times. Place tube in instrument and make scale reading.

When oxalated blood is available, a 1:250 dilution is made by adding 0.1 ml. of blood to a solution of dilute NH_4OH. Rinse pipette, oxygenate and make up to 25 ml. with reagent. Pour approximately 5 ml. in Klett-Summerson tube and read as above.

Calculation.

$$\frac{\text{Reading of unknown}}{\text{Scale reading corresponding to 1 gram of hemoglobin*}} = \text{grams hemoglobin per 100 ml. blood}$$

Calibration of Instrument. The hemoglobin content of a sample of well mixed oxalated or heparinized blood is determined by the iron concentration method. Duplicate aliquots of this same blood are diluted 1:250 as directed under the oxyhemoglobin method and read on the photoelectric instrument using the green filter. This scale reading is designated as Roxy below.

$$\frac{\text{Roxy}}{\text{gm. of hemoglobin by Wong method}} = \text{scale reading corresponding to 1 gram of hemoglobin.}$$

If preferred a factor can be obtained as follows:

$$\frac{\text{Grams of hemoglobin by Wong method}}{\text{Roxy}} = \text{hemoglobin factor}$$

Reading of the unknown $\times$ hemoglobin factor grams of hemoglobin per 100 ml.

Acid Hematin Methods. The red blood cells are laked in dilute hydrochloric acid. The resulting brown and relatively clear acid hematin solution is matched with standards.

The Sahli Hemoglobinometers. Instruments which utilize a tube in which the acid hematin solution is diluted to match a standard are collectively known as Sahli instruments. Various manufacturers have modifications of this instrument, such as the Sargent-Sahli, Hellige-

* As determined by iron concentration method. See below.

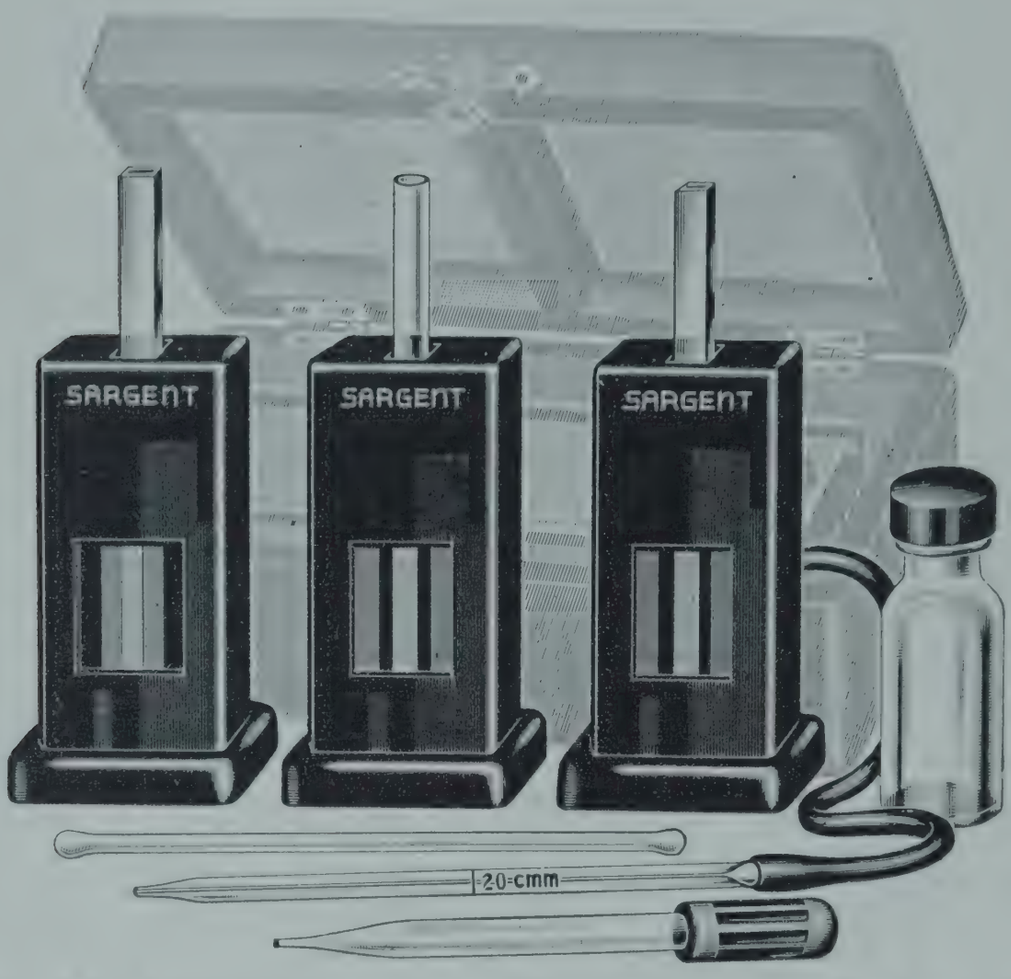


FIG. 1.40. Sahli hemoglobinometers. This instrument utilizes an acid-hematin mixture after conversion by the addition of hydrochloric acid. Courtesy E. H. Sargent Co.

Sahli, Sahli-Haden, etc. (Fig. 1.40). Square measuring tubes with plano-parallel walls are preferred to cylinders.

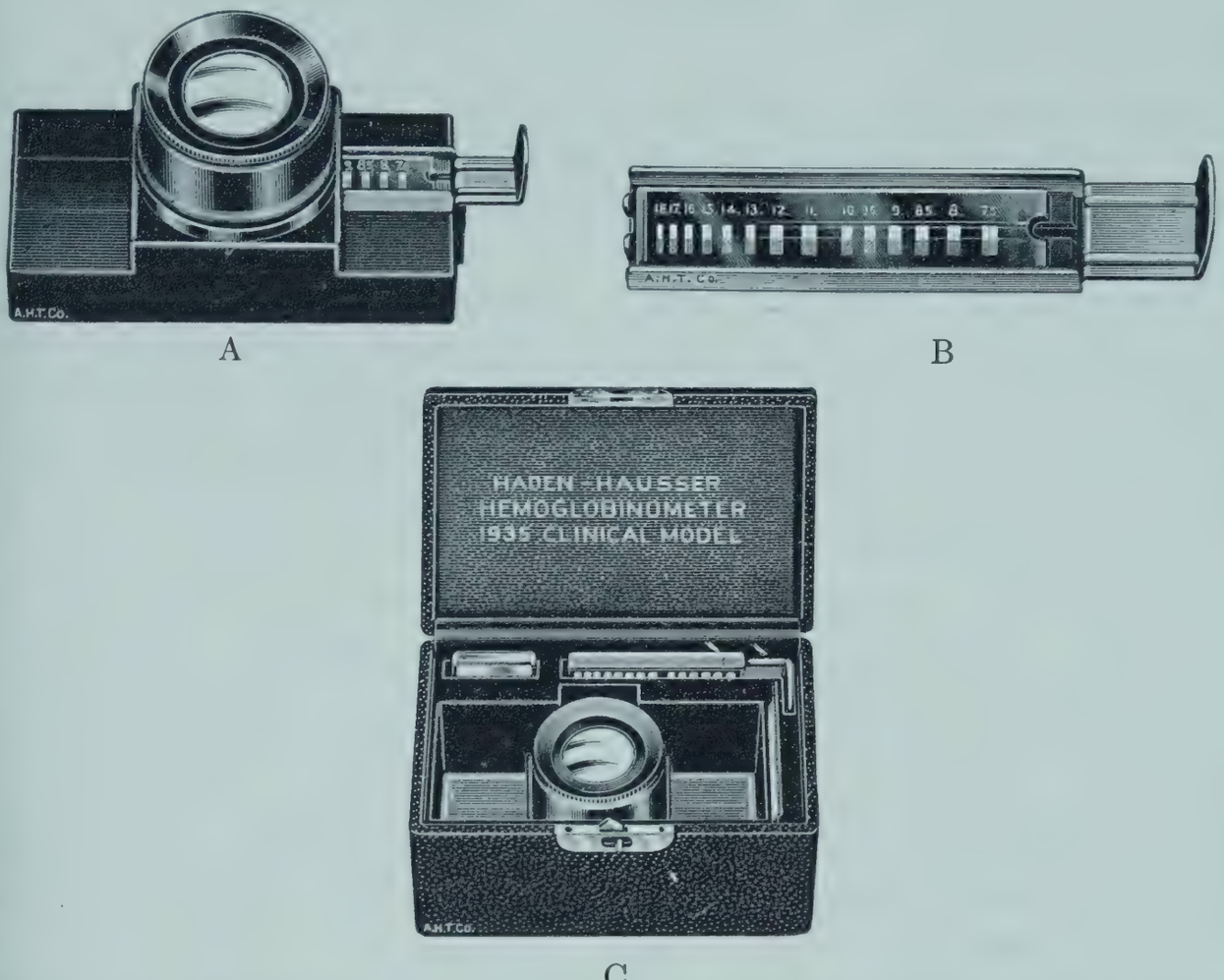
Approximately 0.5 ml. of N/10 HCl is placed in a graduated tube. Blood is drawn up to the 20 cu. mm. mark in a special capillary pipette. The excess blood is wiped from the tip and the blood is discharged into the acid solution after which the pipette is rinsed several times. After allowing the mixture to stand 20 minutes or longer for the maximal color to develop, the acid solution is diluted with water or dilute HCl, mixing after each drop with a glass rod or by tilting the tube until the solution in the tube matches that of the color standard. The height of the column at which a match is obtained determines the value of hemoglobin in grams per 100 ml.

The Sahli instruments are not suitable for laboratories performing multiple tests each day. A common error is to substitute a cylinder for the original one furnished by the manufacturer and to assume that it will give reliable readings. Each cylinder must be standardized for a given standard and the suitable correction factor used for each cylinder.

The Haden-Hausser Instruments. Acid hematin solution of known

dilution is placed in a wedge shaped channel and the intensity of the color matched with a glass standard scale through a magnifying glass.

Two instruments are available. The smaller and less expensive instrument is known as the clinical model (Fig. 1.41). A larger model with a built in light source, known as the laboratory model, is designed for larger laboratories performing multiple tests.



A B C

FIG. 1.41. The Haden-Hausser hemoglobinometer. This utilizes the principle of conversion of hemoglobin into acid-hematin by the addition of hydrochloric acid. A. Front view, showing comparator slide in position. B. Comparator slide, with cover glass in position. C. Complete outfit in case.

Blood is drawn up to the 0.5 mark in the white cell pipette and N/10 HCl to the 11 mark. The pipette is shaken and the mixture allowed to stand 20 or more minutes.

A coverslip is placed on the comparator slide with the beveled side down and with the lower and left edge in contact with the metal frame. The first few drops of fluid in the pipette are discarded and the channel filled with the acid hematin solution. The carrier is placed in a holder and examined in a good light. The slide is moved back and forth to determine the point at which there is the best color match.

One of the advantages of the Haden-Hausser method is that it is com-

pact and convenient and therefore it is popular with medical students and practitioners. With the clinical model it is possible to perform a white cell count and hemoglobin estimation with the fluid from the same pipette.

The accuracy of this instrument is said to be comparable to the Sahli instruments in which the fluid is diluted. There is more personal variation in color matching with the Haden-Hausser instrument, and it is difficult to match the colors in the higher scale readings. The lowest reading possible on the instrument is 7.5 gm. Therefore when severe anemia is suspected the blood should be drawn to the 1.0 mark instead of the 0.5 mark and the reading divided by 2.

Photoelectric Method.

Disadvantages of the acid hematin methods are that the color does not develop to a maximum immediately and the color intensity is not directly proportional to the hemoglobin concentration when the values are abnormally high or low. Photoelectric acid hematin methods are inferior to oxyhemoglobin and cyanmethemoglobin methods.

Cyanmethemoglobin Method. The cyanmethemoglobin method is widely used and is considered to be superior because cyanmethemoglobin is the most stable of the various hemoglobin pigments. Many have feared to adopt the procedure because of the use of a reagent containing cyanide. This objection is not valid if cyanide is treated with the same caution as concentrated acids and pathogenic bacteria. Standard solutions for this procedure have recently been made available to the Armed Services (Crosby, Munn and Furth: U. S. Armed Forces M. J. 5: 693, May, 1954).

Tallqvist Method. A drop of blood is placed on a piece of white absorbent paper and allowed to dry for a few seconds. The paper is folded and the drop inserted under the central perforations of a serial red color chart (Fig. 1.42). The intensity of the red color of the drop is matched with the most suitable color on the scale and the hemoglobin read as the value which this red scale represents.

The method is simple and inexpensive. It has a place as a screening procedure in situations where more accurate methods are not available as in the examination of applicants for employment, donors for transfusion, in home practice and in emergency situations where the staff is inadequate to cope with the patient load. The error of the method is great for it is impossible to control the thickness of the drop, the color scale is not accurately calibrated and there are differences in color depending on the amount of moisture. The Tallqvist method can be used

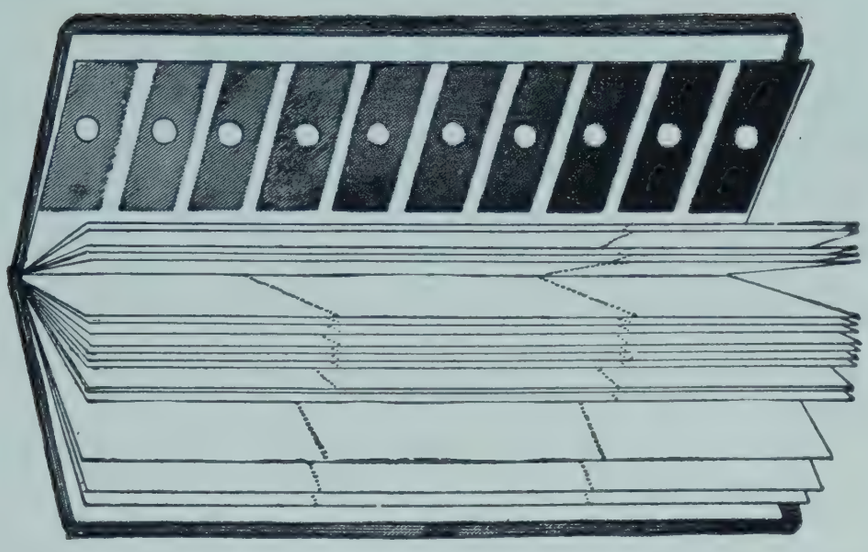


FIG. 1.42. A Tallqvist hemoglobin scale. A drop of blood is placed on a piece of blotting paper and compared with the variations in the red scale in the back of the book.

to tell whether a patient needs to have an anemia study but should never be used to determine mean corpuscular values, to determine what kind of antianemic therapy is most suitable or to evaluate the response to therapy.

Normal Values

The normal value for hemoglobin for adult males varies between 14 and 18 gm. with the average approximately 16 gm. per 100 ml. The normal for women varies between 12 and 16 gm. with the average at approximately 14 gm. per 100 ml.

The newborn child has a high hemoglobin concentration, but shortly after birth and during childhood the normal hemoglobin values are below adult levels. For average values at various ages see Table 1.3. Elderly people as a class have lower values than do younger adults. At all age groups there is considerable physiological variation, which taken together with the known errors of the commonly used methods should make one hesitant to consider as significant variations of a few grams above or below the average value for a given age and sex.

THE HEMATOCRIT

One of the most accurate, simple and inexpensive methods for the detection and measuring the degree of anemia is the estimation of the volume of packed red blood cells. This is done by centrifuging blood until there is maximal packing of the cells.

The method is particularly suitable as a routine and screening procedure and for use in general practice. Blood can be drawn by a nurse or office assistant at the time the blood for serological testing is collected.

TABLE 103

*Average Normal Blood Values at Different Age Levels**

	At birth	At 2 days	At 14 days	At 3 months	At 6 months	At 1 year	At 2 years	At 4 years	At 8–12 years
Red cells per cu. mm. (in millions)	5.1	5.3	5.0	4.3	4.6	4.7	4.8	4.8	5.1
Hemoglobin (grams per 100 ml.)	17.6	18.0	17.0	11.4	11.5	12.2	12.9	13.1	14.1
White cells per cu. mm. (in thousands)	15.0	21.0	11.0	9.5	9.2	9.0	8.5	8.0	8.0
Platelets per cu. mm. (in thousands)	350.0	400.0	300.0	260.0	250.0	250.0	250.0	250.0	250.0
Differential smears (percentages):									
Polymorphonuclear neutrophil	45	55	36	35	40	40	40	50	60
Eosinophil	3	5	3	3	3	2	2	2	2
Lymphocyte	30	20	53	55	51	53	50	40	30
Monocyte	12	15	8	7	6	5	8	8	8
Immature white cell	10	5	—	—	—	—	—	—	—
Percentage of nucleated red cells in total nucleated cells	1–5	2	—	—	—	—	—	—	—
Percentage of reticulocytes in total red cells	2	3	1	0.5	0.8	1	1	1	1

* Ref.: Atlas of the Blood in Children, Blackfan, K. D., and Diamond, L. K., Commonwealth Fund, New York, 1944.

The major work is done by the centrifuge. No special technical skills or standardization of equipment are required.

If the Wintrobe method is used, it is possible to obtain from the same specimen of blood other valuable information, for the sedimentation rate can be determined before the tube is centrifuged, the color of the supernatant plasma will give indications of jaundice, hemolysis or lipemia and the buffy coat of white cells on top of the red cell column will be a rough index of the white cell count. One millimeter of buffy coat represents approximately 10,000 white cells, provided the distribution of cell types is fairly normal.

Any type of graduated centrifuge tube may be used. Blood may be drawn into a capillary tube coated with heparin, one end sealed and the height of the red cell column after centrifugation measured with an

ordinary ruler. The methods most used in this country utilize the Wintrobe and the Sanford-Magath tubes.

Centrifuges with a conical head are efficient in spinning the cells down, but have the disadvantage of yielding a slanted top to the red cell column. The approximate level can be estimated without significant error as midway between the upper and lower level.

Methods

Wintrobe Method. The Wintrobe tube is a stout narrow glass tube which is approximately 11 cm. long, with a flat bottom, internal diameter of 2.5 mm., and a volume of approximately 1 ml. It is graduated in millimeters with a double row of numerals at intervals of 1 to 10 cm. The graduations on the right are used for determining the volume of packed red blood cells and on the left for the sedimentation rate (Figs. 1.43 and 1.44).

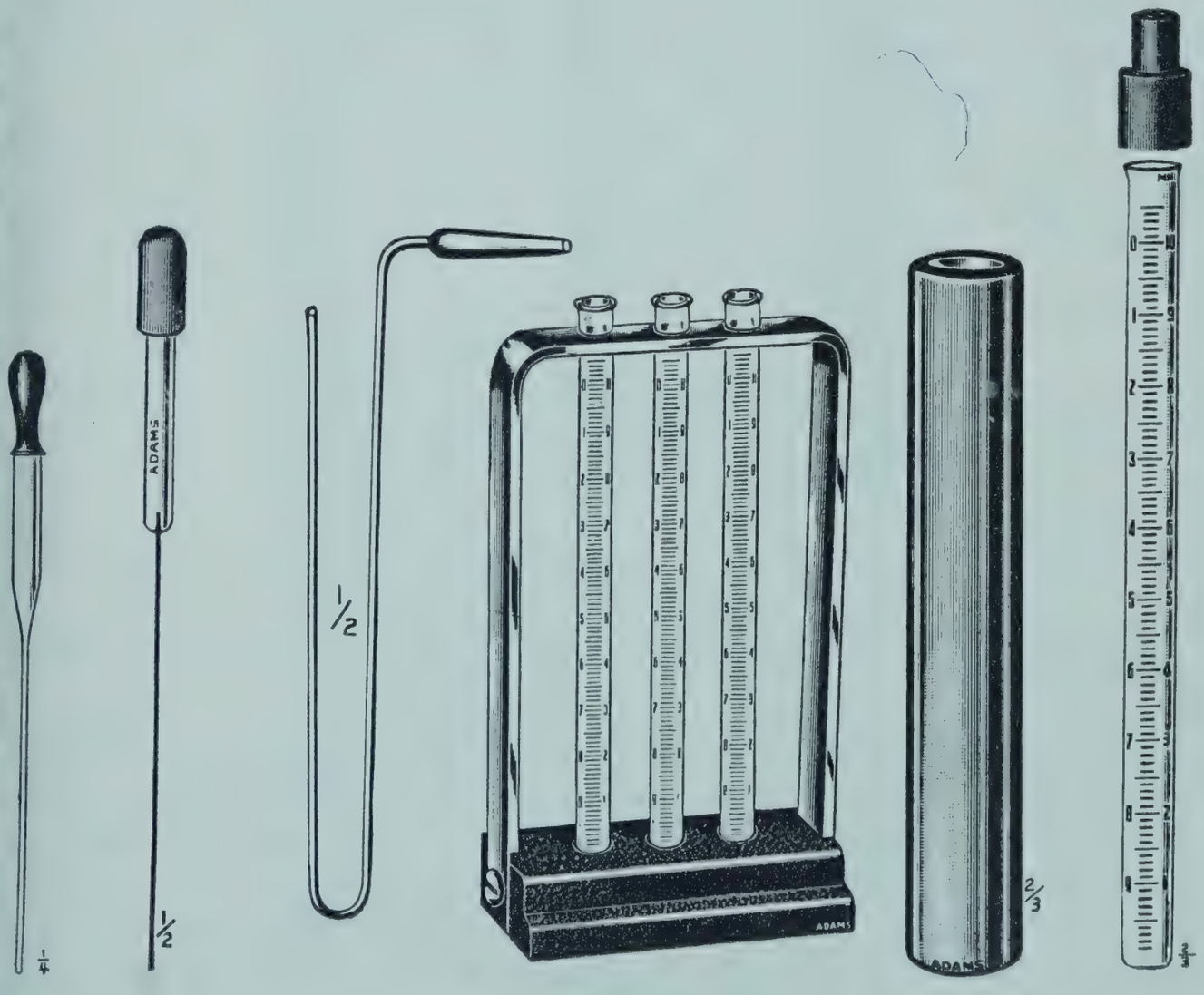


FIG. 1.43. Wintrobe hematocrit tube and accessories. Left to right, pipette, all glass; pipette with stainless steel capillary; tube cleaner; Wintrobe rack; adapter for centrifuge shield to hold Wintrobe tube; Wintrobe hematocrit and sedimentation tube with rubber cap. Courtesy of Clay Adams Co., Inc., N. Y.

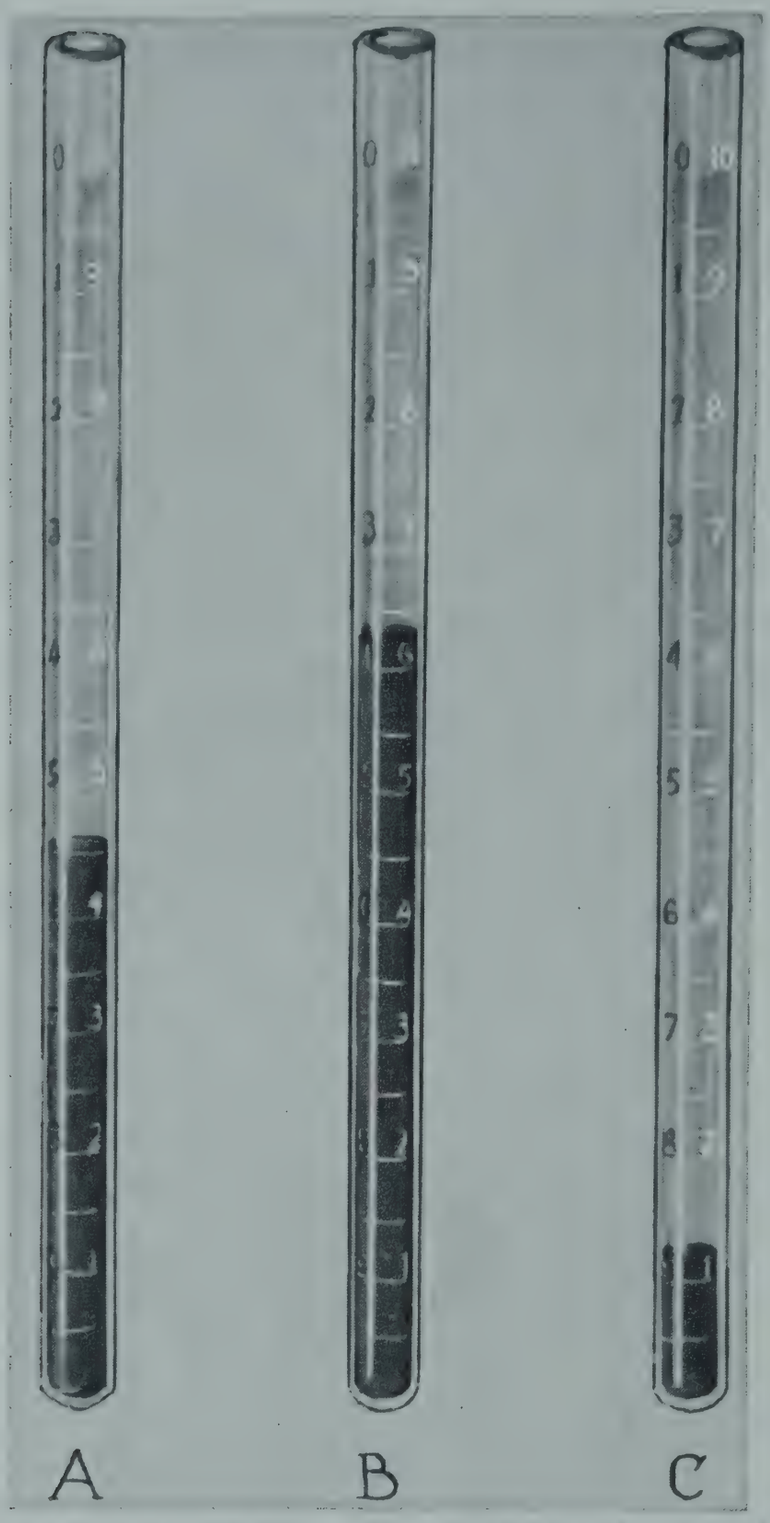


FIG. 1.44. Showing hematocrit tubes after cells have been packed down in (a) normal blood, (b) erythrocytosis, (c) anemia.

Before use the tubes must be clean and dry. Cleaning is facilitated by use of U-shaped metal tubes attached to a suction device. The Wintrobe tube is inverted over one end of the "U" with the open end of the tube immersed in water.

For the filling of the tube, a long narrow metal cylinder attached to a medicine dropper or a 5 inch, 13 gauge needle attached to a syringe is preferred. Lumbar puncture needles may be used. Glass pipettes small and long enough to go into the Wintrobe tube are too fragile to be practical.

For routine use it is recommended that 5 ml. of venous blood be drawn and added to ammonium and potassium oxalate (see under Anticoagulants, page 3). Adequate mixing is essential.

Mix blood again immediately before drawing it up into the pipette. Place tip of pipette in bottom of Wintrobe tube and fill from the bottom, raising the pipette as the blood goes in to avoid air bubbles. Adjust level of blood to 0 mark. Place tube in centrifuge cup and spin at 3,000 or more r.p.m. until there is a constant pack. This can be determined by making readings at 15 minute intervals. The time required can be determined by trial runs for each centrifuge speed and length of centrifuge arm. The reading is made in terms of ml. of packed red blood cells per 100 ml.

$$\frac{\text{Height of red cell column (mm.)}}{\text{Total height of column (mm.)}} \times 100 = \text{Volume per cent of packed red blood cells}$$

Micro-Hematocrit Methods. The determination of the volume of packed red blood cells can be accurately and rapidly performed on capillary blood using heparin coated capillary tubes and a high speed centrifuge designed for this purpose (Fig. 1.46).

Blood from a skin puncture wound is taken directly into a heparinized capillary tube, 75 mm. long with a diameter of 1.2 to 1.5 mm. Duplicate

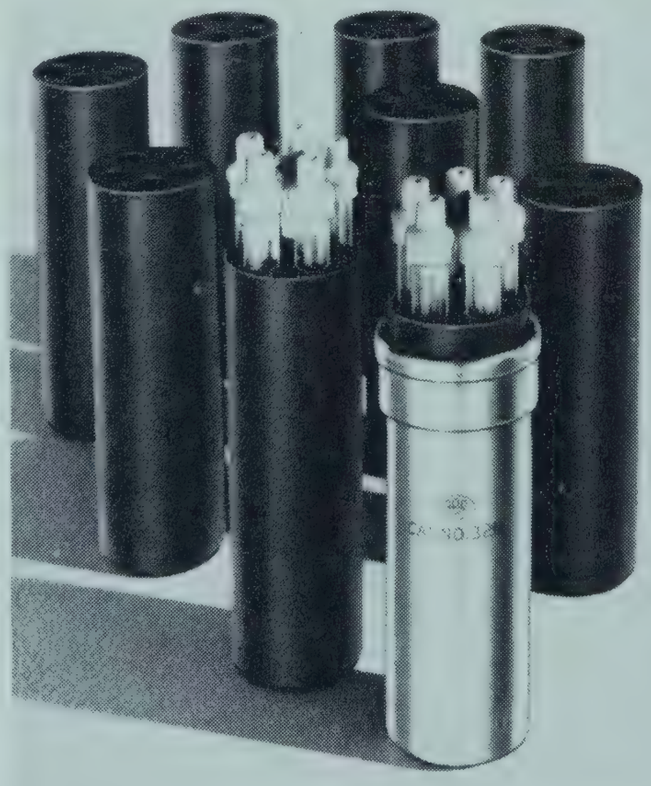


FIG. 1.45. Multiple carrier to accommodate Wintrobe hematocrit tubes (Courtesy of International Equipment Company, Boston, Mass.).



FIG. 1.46. High-speed micro-hematocrit centrifuge for capillary blood tubes (Courtesy of International Equipment Company, Boston, Mass.).

tubes are filled for each patient. The tube is filled to approximately 1 cm. from the end. The tubes may be transported by placing in the slots of a standard slide box. The number in the bottom of the slide box corresponds to the number assigned to a given patient. The end of the tube which does not contain blood is sealed by means of a "Microblast burner" (A. H. Thomas ✻2641) which provides a hot, needle point flame. The two tubes for a given patient are placed opposite each other in the slots in the centrifuge head. The top is fastened and the lid closed. The centrifuge is started and allowed to spin at top speed for 3 to 5 minutes, depending on the speed of the centrifuge. After centrifugation, the tubes are removed and the column of packed red cells read by means of a graphic chart (Fig. 1.47) which makes it possible to read the height of the red cell column in terms of per cent of original whole blood column. A magnifying glass and an illuminated box aid in making the readings.

The micro-hematocrit method is particularly useful in children and in the management of patients with burns and in shock. It is an ex-

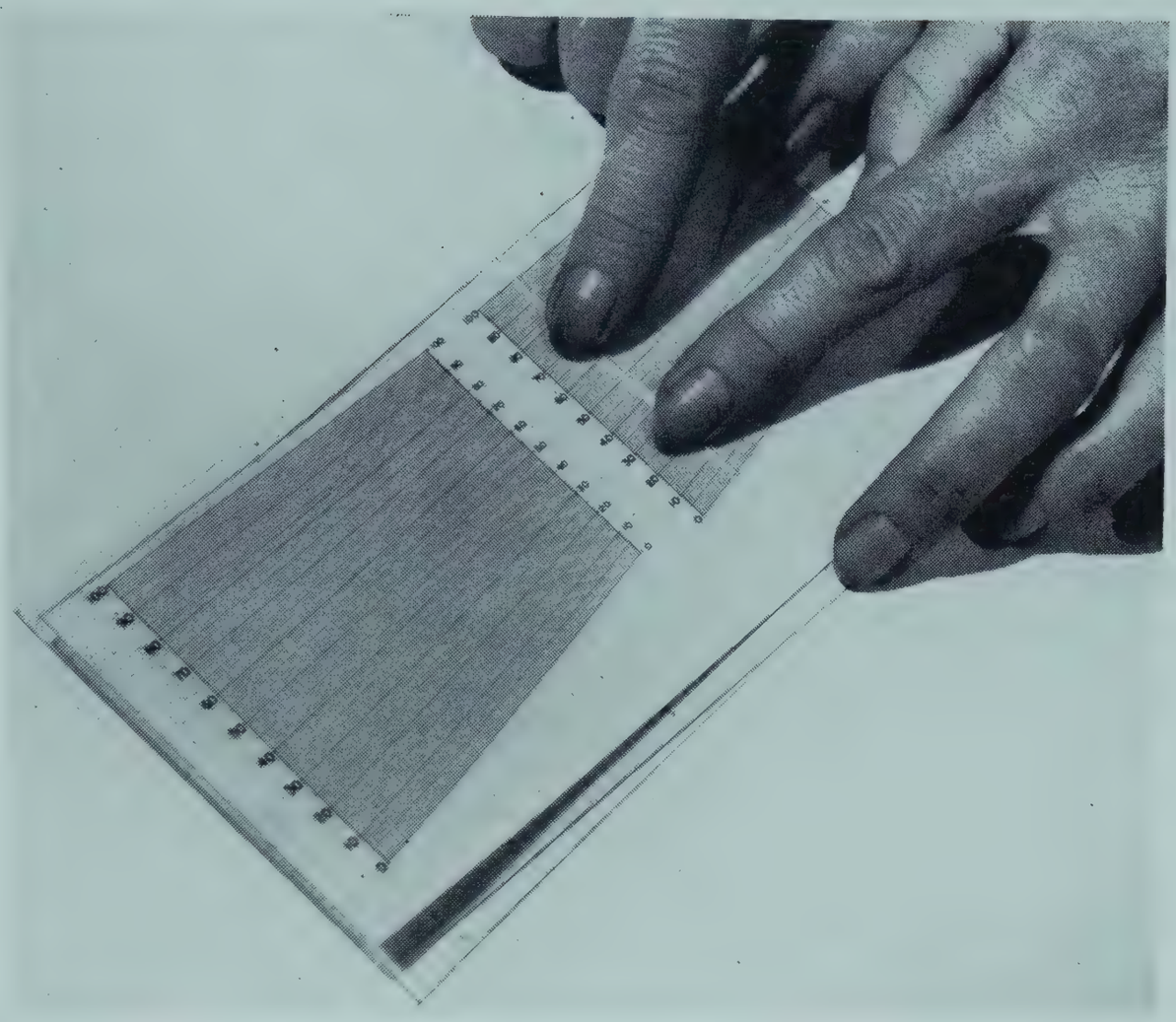


FIG. 1.47. Graphic reader—micro-hematocrit method, graduated 0 to 100% cells (Courtesy of Aloe Scientific Company).

cellent method to use as a screening procedure for anemia and as a routine test in place of red cell counts and hemoglobin estimations. The method yields results which are comparable to those obtained by the Wintrobe or larger graduated tube methods.

Normal Values

The normal hematocrit for men varies from 40 to 50 ml. (average 45) and from 35 to 45 for women (average 42). For children see Table 1.3.

Values above 55 and below 40 for men and above 50 and below 35 in women are significant of abnormality. In severe anemias the packed cell volume may be less than 10 ml. per 100, and in erythrocytosis it is often above 60.

The error of the hematocrit methods is ± 2 to 4 per cent.

MEAN CORPUSCULAR VALUES

Having determined the red cell count, hemoglobin concentration and volume of packed red blood cells in a given blood specimen, it is possible

to calculate the volume and hemoglobin content of the average red cell and the concentration of the hemoglobin in the red cell mass. These calculations are of value in determining the types of anemia and in guiding therapy (Chapter 3).

Mean Corpuscular Volume is determined by dividing the volume of packed erythrocytes per 1000 ml. of blood by the red cell count in millions per cubic millimeter. The result is expressed in cubic microns.

$$\text{M.C.V.} = \frac{432}{5.0} = 86.4 \text{ cubic microns}$$

The average values for mean corpuscular volume in normal blood lie between 80 and 100 cubic microns, the theoretical normal being 86.4.

Mean Corpuscular Hemoglobin is determined by dividing hemoglobin in grams per 1000 ml. of blood by the red cell count in millions per cu. mm. The result is expressed in micromicrograms.

$$\text{M.C.H.} = \frac{145}{5.0} = 29 \text{ micromicrograms}$$

The average values for mean corpuscular hemoglobin in normal blood range from 27 to 32 micromicrograms, the theoretical normal being 29.

Mean Corpuscular Hemoglobin Concentration is computed by dividing hemoglobin in grams per 10,000 ml. of blood (or hemoglobin in grams per 100 ml. $\times$ 100) by the volume of packed red cells per 100 ml. of blood. The result is expressed as per cent of hemoglobin of the total weight of an erythrocyte.

$$\text{Mean Corpuscular Hemoglobin Concentration} = \frac{1450}{43.2} = 33.6\%$$

Average values in normal blood fall between 33 and 38. The theoretical normal is usually considered to be 34 per cent.

It is thought that the hemoglobin concentration in the normal red cell is maximal and that concentration above normal is impossible. Thus a mean corpuscular value above 40 per cent is probably due to technical errors in hemoglobin and/or hematocrit. The only exception to this rule occurs in sickle cell anemia. This is possibly due to the loss of water associated with the molecular rearrangement of hemoglobin in the sickled cell.

Mean corpuscular values are preferred to the various indices, such as volume index, color index, and saturation index, for the mean corpuscular values utilize absolute figures while the indices deal with the relationship of arbitrary values.

SEDIMENTATION RATE

When whole blood mixed with an anticoagulant is placed in a perpendicular tube, the red cells sink because they are heavier than the plasma in which they are suspended. The speed of fall of the blood of normal individuals, in which the red cells are well separated, is relatively slow; but in inflammatory diseases and diseases in which there is tissue degeneration and necrosis, changes occur in the physico-chemical properties of the plasma and the erythrocyte surfaces which cause the red cells to aggregate or to form rouleaux. The surface area of the erythrocyte clumps is less in relation to the total volume than is the combined surface area of the individual cells and as a consequence the cell aggregates have an increased rate of fall. The larger the agglomeration the faster the sinking velocity. Also the aggregation of cells creates wide spaces between the cells and cell groups, which allows the displaced plasma to rise with less retarding effect as the cells sink.

The principal factors influencing the sticking together of the red cells and therefore the sedimentation rate are the concentrations of the various proteins. A relative or absolute increase in fibrinogen or globulin is associated with an increase in sinking velocity, whereas an increase in albumin causes the cells to fall more slowly.

Macrocytosis and spherocytosis of the red cells, hypercholesteremia and decrease in the number of red cells in relation to the concentration of plasma proteins cause an increase in fall, whereas microcytes, leptocytes and poikilocytes and increased lecithin tend to retard settling. Women as a rule have higher rates than men. Changes associated with the menstrual cycle are not significant, but there is a progressive increase in sedimentation rate after the first trimester of pregnancy with a return to normal values a few weeks after delivery. Elderly people as a class have slightly faster rates than do younger individuals. The rates for children are essentially the same as for adults.

When the distance traversed by the red cells from the top of the blood column is plotted against time, a sigmoid shape of curve is obtained. During the first few minutes the cells are beginning to form rouleaux and fall relatively slowly (phase of aggregation). As the aggregates become larger the rate of fall becomes progressively faster (phase of maximal fall) until the cells begin to pack (phase of packing) after which the rate of fall is progressively slower.

Numerous tubes, methods and modifications of methods have been devised for determining the falling velocity of erythrocytes. The most accurate are those such as the Rourke-Ernstene and the Cutler modifica-

tion, for in these methods the rate during the intermediate phase of most rapid fall is recorded.

For clinical purposes the simplest, and one of the most reliable methods, and one which lends itself well to multiple tests is the Westergren. The Wintrobe and Landsberg procedure is also recommended and widely used because it is practical, utilizes a small amount of blood and because a study of the hematocrit, buffy coat and character of the plasma can be made with the same tube of blood. In the latter two methods the readings are made at the end of an hour.

Correction for Anemia

One of the controversial points about the sedimentation rate concerns the correction for anemia. Because anemia and increased sedimentation rates are often associated, and because it is known that the sedimentation rates of red cells diluted with plasma from the same individual fall faster than do the undiluted cells, attempts have been made to correct the sedimentation rate for anemia. Various reference charts based on the hematocrit have been developed, the ones most used being the Rourke-Ernstene, Wintrobe and Landsberg, and the Hynes and Whitby. Many physicians think that the correction for anemia is essential, whereas a minority question the advisability and accuracy of such corrections.

Reasons for not correcting the anemia are: 1. In certain anemias (hypochromic microcytic, sickle cell anemia, and anemias characterized by marked poikilocytosis) the sedimentation rate is not increased. 2. In numerous patients with anemia and tissue changes known to be associated with an increased sedimentation rate such as active tuberculosis, the sedimentation rate is abnormal, but when corrected for anemia, normal values are obtained. 3. The correction chart of Wintrobe and Landsberg is based upon manipulations of normal blood and is not applicable over the entire range of observed hematocrit values and sedimentation rates. 4. In a given patient, the sedimentation rate and/or degree of anemia may change without correlation.

In the author's opinion the correction for anemia is not accurate, is not necessary, is often misleading and may mask significant pathology.

Because of the difference of opinion concerning the correction for anemia, it is advised that the sedimentation rate be reported as it is observed, stating the method used, and that the so-called "corrected sedimentation rate" be also reported, stating the method used for this manipulation. The clinician is then free to interpret as he chooses.

Methods

Westergren Method. The Westergren pipette has 2 mm. bore, a capacity of 1 ml., a total length of 300 mm. and graduations from 0 to 200 at 1 mm. intervals. Special racks with spring clips are available for holding the lower end of the pipette tightly against a rubber mat in a vertical position (Fig. 1.48).

4.5 ml. of venous blood are mixed with 0.5 ml. of 3.8 per cent aqueous sodium citrate. The pipette is filled to the 0 mark and placed in an upright position in the rack. The fall of the erythrocytes in millimeters in 1 hour is noted. The normal sedimentation rate with this method is



FIG. 1.48. Blood sedimentation apparatus—Westergren. Compliments of the A. S. Aloe Co., St. Louis, Mo.

0 to 15 mm. for men with an average of 4 mm., and 0 to 20 mm. for women with an average of 5 mm.

The advantages of the Westergren procedures are that the pipettes are easy to fill and to clean and the error due to the packing of red cells in 1 hour's time is minimized because of the length of the tube.

Rourke-Ernstene Method (modified). The Rourke-Ernstene tube is 12 cm. long, has an internal diameter of 4 mm. and is calibrated at 2 mm. intervals. It is preferred to the Wintrobe tube because it has a wider lumen, making it easier to fill and to clean. It is longer than the Wintrobe tube and thus the error due to packing is reduced. 0.06 ml. of an anticoagulant mixture containing 2 gm. of potassium oxalate and 3 gm. of ammonium oxalate in 100 ml. of distilled water are placed in a vial and evaporated to dryness in an incubator. 1.5 ml. of venous blood

are placed in the stoppered vial. After thorough mixing of blood with oxalate, the Rourke-Ernstene tube is filled by means of a pipette to the 0 mark.

The tube is placed in an upright position or suspended from a rack made by cutting holes in a rigid metal plate through which the Rourke-Ernstene tubes are inserted.

Four readings are made at 15-minute intervals. The sedimentation rate during the period of most rapid fall is taken as most representative The rate is expressed as "millimeters fall per minute." Thus if there were a 15 mm. fall in 15 minutes, the rate would be 1 mm. per minute.

After the sedimentation rate has been determined, the tube is placed in a centrifuge and the volume of packed red cells determined. A correction for anemia is determined by reference to a chart (Fig. 1.49).

This is done by finding the junction of the lines representing the observed rate of fall per minute and the observed hematocrit reading. The sloping line corresponding to this point is followed until it intersects the 45 per cent hematocrit line. A horizontal line extending to the left from this latter point determines the corrected sedimentation rate. Example: Observed sedimentation rate 3.8 mm. per minute, hematocrit 25 vol. per cent. Corrected rate is 1.5 mm.

Wintrobe and Landsberg Method. 5 ml. of venous blood are drawn in a vial containing 6 mg. of dried ammonium oxalate and 4 mg. of dried potassium oxalate. The blood is mixed and the Wintrobe tube filled to the 0 mark (Fig. 1.43). Air bubbles are avoided by filling the tube from the bottom and withdrawing the pipette as the tube is filled. The tube is placed in an upright position and the amount of settling in millimeters noted at the end of one hour.

The tube is placed in a centrifuge and spun to a constant volume. The corrected sedimentation rate is then computed by means of a graphic chart (Fig. 1.50).

The normal sedimentation rate with this method is 0 to 9 mm. per hour for men and 0 to 20 mm. for women.

Technical Factors

One of the principal sources of error in the determination of the sedimentation rate is the tilting of the tube, which causes a significant increase in the falling velocity of the cells. This is explained by the fact that the cells settling in a slanting tube begin to pack all along the lower side of the tube. Also, the cells near the upper side of the tube

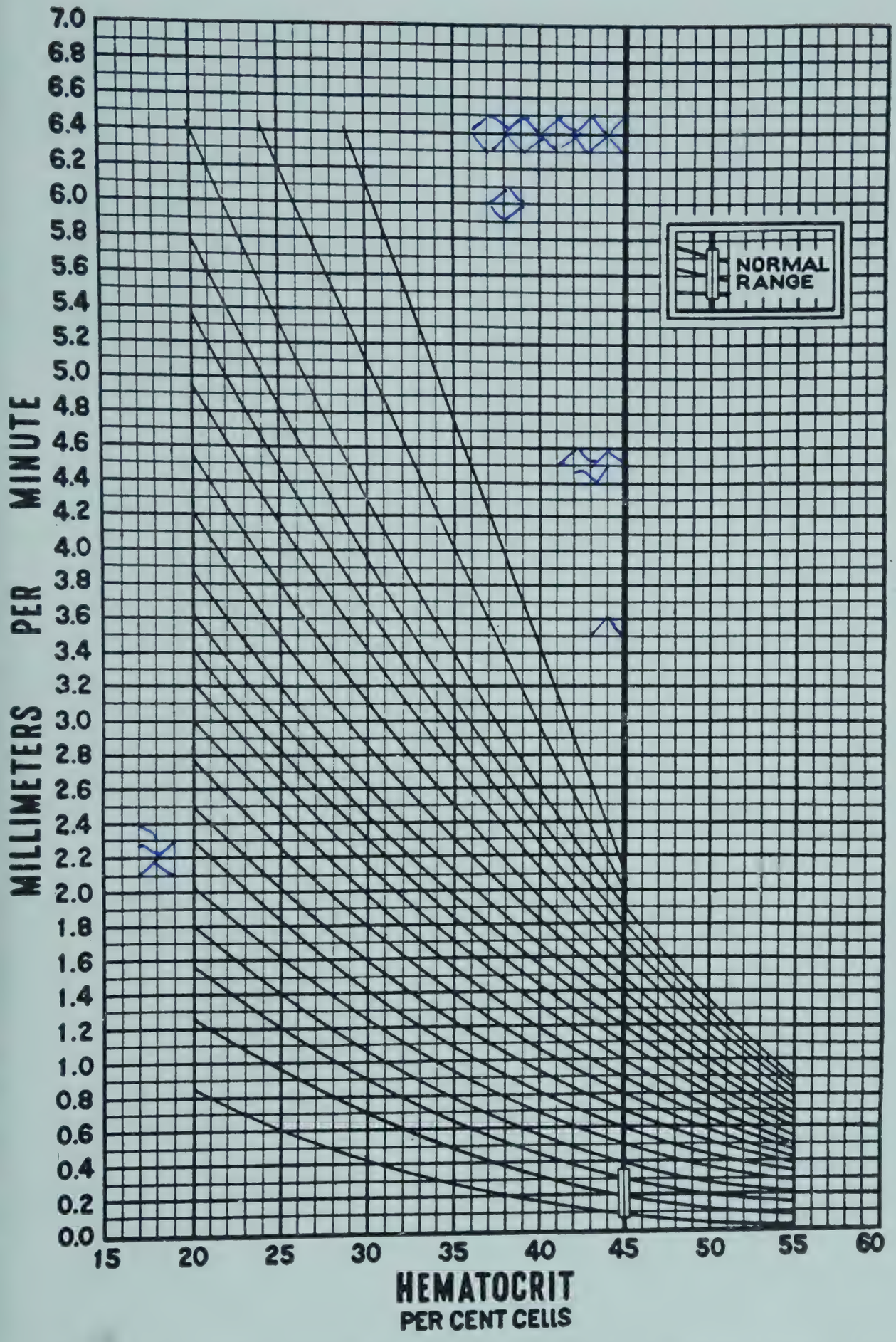


FIG. 1.49. Rourke-Ernstene chart for correction of sedimentation rate.

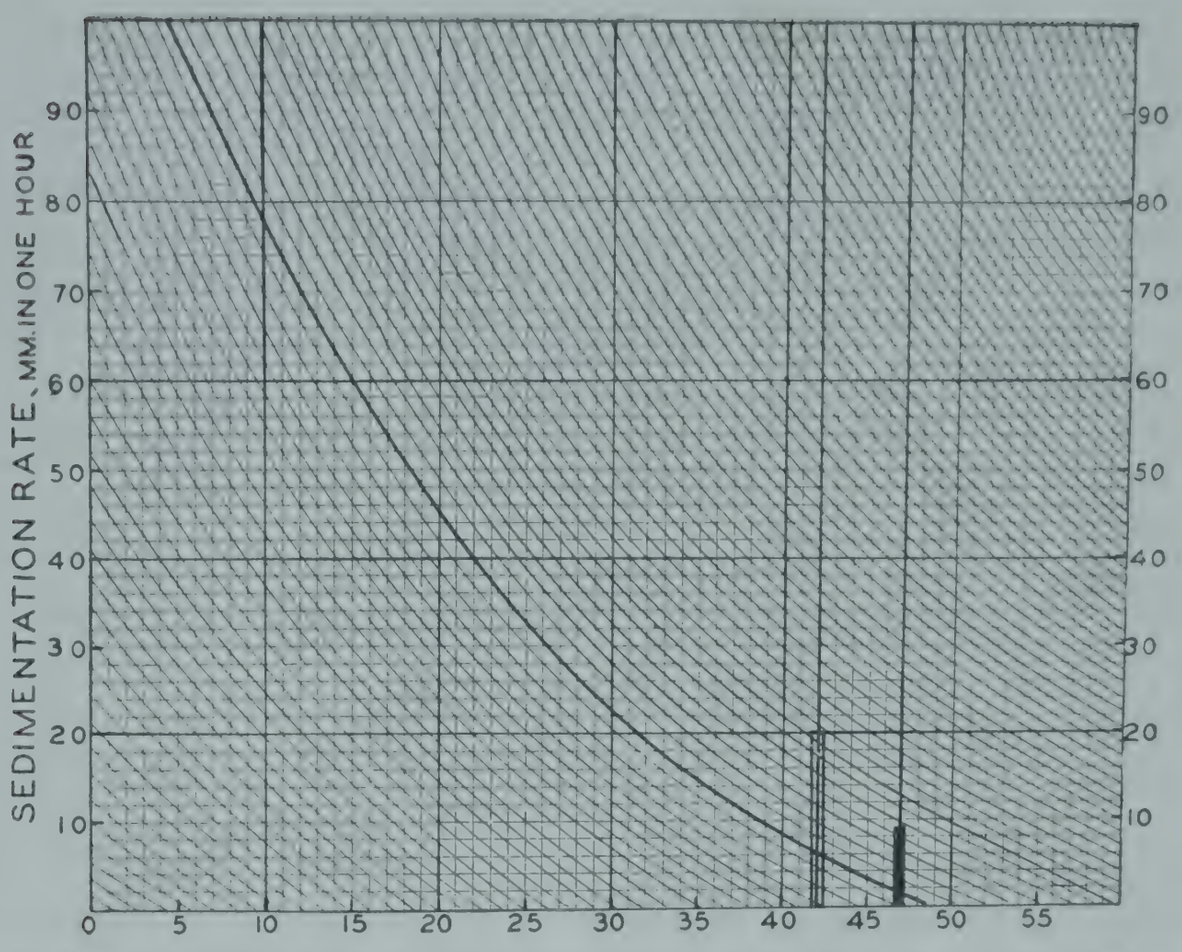


FIG. 1.50. Correction chart for sedimentation time from Wintrobe and Landsberg. Use of chart: First, find the horizontal line that corresponds to the mm. of sedimentation for 1 hour. Then, follow this line until it intersects the vertical line representing the volume of packed red cells. At this point, follow the nearest curved line downward until it intersects the dark line at 42, if the patient is a woman; if a man, follow the curved line until it intersects the dark line at 47. This point of intersection represents the corrected sedimentation rate, when read on the horizontal line. From Am. J. Med. Sc., January, 1935.

rapidly get out of the way so that the displaced plasma on rising is not opposed by falling cells as it is in tubes in the upright position.

The sedimentation rates of blood in tubes of different lengths are comparable provided the readings are made during the period of maximal fall, but there are gross differences in the readings when short and long tubes are used and the readings are made in 1 hour, for the packing phase comes much sooner in short tubes and the error due to variations in packed cell volume is magnified.

The test should be performed within several hours after collection for on standing the cells tend to become spherical and do not form rouleaux as readily as when they are fresh. Care must be taken, not only to mix the blood thoroughly before filling the sedimentation tube but to aerate it well, for an increase in carbon dioxide retards the rate of fall.

Other factors which favor retardation of the falling velocity are de-

fibrination, partial clotting of the blood, low temperature, excess dry anticoagulation salt, dilution of the blood with aqueous anticoagulants and use of capillary tubes with a diameter less than 2 mm.

Clinical Applications

The sedimentation rate is increased in any disease in which there is inflammatory reaction, tissue degeneration, suppuration or necrosis. The sedimentation rate is usually not altered in small focal infections, in the first few hours of an acute infection as in an acute appendicitis, in ulcerations of the mucous membranes in which there is little inflammatory reaction and in many skin lesions.

In the majority of non-infectious allergic conditions, nutritional deficiency states, metabolic diseases, simple cachexia, uncomplicated hypertension and compensated heart disease, and in the minor respiratory infections, the sedimentation rate may not be significantly altered.

The sedimentation rate finds useful application in following the progress of a given established lesion in such diseases as tuberculosis, chronic infections, rheumatic fever, coronary thrombosis, the arthritides and nephritis. A rapid rate is indicative of an active lesion and an increasing rate of an extension of the process, whereas a subsiding rate is indicative of improvement. Since the sedimentation rate is often more sensitive than the temperature, weight, white cell count or subjective symptoms the test is of value in guiding therapy, surgical procedures and convalescence.

The sedimentation rate is useful as a screening diagnostic procedure for abnormalities are often revealed before there are evidences of localization. The test is useful in differentiating functional states and neuroses from organic disease. A normal sedimentation rate does not exclude organic disease, but an increased rate in a person considered to have a neurosis should lead to further study and reconsideration of symptoms. A marked increase in rate is significant, but weight should not be given to a slight or doubtful increase. In industrial medicine the sedimentation rate is of value for it furnishes objective evidence of abnormality and helps in making decisions about muscle and joint aches, fatigue and other vague and indefinite subjective symptoms. A normal sedimentation rate gives the physician reassurance in telling the patient that there is no apparent serious organic disease.

The sedimentation rate is helpful in differentiating rheumatoid arthritis and gout from osteoarthritis, for in the latter condition the sedimentation rate is often normal. The sedimentation rate is an unreliable

test in detecting early malignancy and in differentiating benign and malignant tumors, but increased rates are the rule in advanced malignancy. Liver diseases are associated with variable rates which would be anticipated since alterations in protein ratios are common in hepatic disorders.

Ham and associates consider the sedimentation rate of red cells as a semi-quantitative test for abnormal concentrations of fibrinogen and serum globulins, and state "determinations of the sedimentation rate of defibrinated blood serves as a crude index of the concentration of serum globulins."

It is obvious from the above statements that the sedimentation test, while simple to perform and to read, is extremely complex—and that the possibilities of technical error are great. Significance should not be attached to minor variations. One of the greatest errors in the use of the procedure has been the tendency on the part of the clinician to interpret the sedimentation rate in the light of the particular disease he is considering at the time, such as rheumatic fever or coronary thrombosis, and to forget other factors. The sedimentation rate is nonspecific and at best roughly quantitative.

THE RETICULOCYTE COUNT

Reticulocytes are immature erythrocytes which in preparations stained with supravital dyes in aqueous solution, such as brilliant cresyl blue, reveal a network of filaments or granules. The granulofilamentous substance is demonstrable in all stages of the nucleated red blood cells and remains demonstrable for a few days after the nucleus is lost and the erythrocytes are delivered into the peripheral blood. The amount, distribution and granularity of the reticular substance vary widely, but in general the filaments are coarser in the earlier cell types and become less distinct as the cells mature.

The granulofilamentous substance within the red cells is not revealed in ordinary blood smears stained with Wright's or other stains in which

Wright's stain in alcohol		Brilliant cresyl blue in aqueous solution
Nucleated red blood cells + Erythrocytes with nuclear fragments or Cabot rings + Diffusely basophilic cells + Stippled cells	=	Reticulocytes

there is primary fixation with alcohol. In general it may be said that erythrocytes which stain blue with Wright's stain would show up as reticulocytes if they could be stained by the supravital dyes without preliminary fixation.

Reticulocytes as a class are larger than non-reticulated cells and are thought to be more resistant to hypotonic salt solutions and to mechanical trauma. They are probably less likely to be phagocytized than are senile cells.

The reticulocyte count is employed as a measure of the physiological activity of the bone marrow.

Methods

Moist Brilliant Cresyl Blue Slide Method. A drop of 1.5 per cent brilliant cresyl blue in 95 per cent alcohol is placed on a warm slide. The drop on drying leaves a thin, fairly homogeneous deposit of dye. After drying the surface is lightly brushed with lens paper to remove coarse particles. Multiple slides may be prepared at one time and stored in a covered slide box until needed.

A small drop of blood is taken on a coverslip and placed drop side down on the film of dye. If the slide and coverslip are clean the drop will spread without pressure, but if it does not spread, gentle pressure may be applied. The edges of the coverslip are rimmed with Vaseline to prevent drying. The preparation is allowed to stand for ten minutes to allow the dye to penetrate the cells, after which 1,000 erythrocytes are counted and the number of erythrocytes, including nucleated red cells that contain reticulum, is noted. The reticulocyte count is usually reported as per cent.

The simplest way to count the reticulocytes is by means of a tally counter which records the total number of erythrocytes. The reticulocytes are totaled mentally or tallied on scratch paper. If a tally counter is not available, the number of erythrocytes in successive oil immersion fields is counted and the number of reticulocytes in the same fields are recorded. An imaginary division of the fields into quadrants aids in accurate enumeration. Various types of ocular discs which limit the size of the visual field increase the accuracy of enumeration. The Miller ocular disc (Bausch and Lomb Optical Co., Rochester, N. Y.) is particularly suitable for this purpose. Unless a special eyepiece is available for reticulocyte counts, it is not recommended that discs be placed in the optical system, for each time the eyepiece is unscrewed dust particles get on the lens system. The trouble created thereby is not worth the advantage gained.

One of the most common errors in making a moist preparation is the use of a drop of blood which is too large; if the drop on spreading touches more than two edges of the coverslip, the preparation is too thick, the cells are not well separated and there is not enough dye in relation to cells to stain adequately. In a satisfactory preparation the nuclei and granules of the white cells and the thrombocytes should be well stained. If they are not, it is assumed there is not enough dye or insufficient time has been allowed. Grease on the slide and coverslip prevents even spreading.

The moist brilliant cresyl blue preparation is useful in checking the number of thrombocytes in thrombocytopenic states. The platelets as seen under oil immersion are readily distinguishable and can be counted in relation to the red cells or white cells. Knowing the number of red cells or white cells the actual number of platelets per cu. mm. can be calculated. The number of thrombocytes obtained by this method is higher than with the direct counting methods, the normal values being 500,000 to 1,500,000 per cu. mm.

Brilliant Cresyl Blue in Saline. A small amount of brilliant cresyl blue (such as can be taken up on the point of a pen knife) is placed in a test tube and approximately 5 ml. of physiological saline added. Two drops of this solution are mixed with one drop of blood on a slide and covered with a coverglass. The excess fluid is removed by pressing a piece of filter paper down on the coverslip. The edges are rimmed with Vaseline to prevent drying. After ten or more minutes the reticulocytes are counted.

If multiple reticulocyte preparations are being performed, a stock solution of 1 gm. of brilliant cresyl blue, 0.4 gm. of sodium citrate and 0.85 gm. of sodium chloride in 100 ml. of distilled water may be prepared. This preparation is unstable and should be filtered before each use.

Dry Brilliant Cresyl Blue Slide Method. Place a drop of 1.5 per cent brilliant cresyl blue in 95 per cent alcohol on a slide and spread with another slide. Allow the film to dry in air.

Make a spread of a small drop of blood on top of the dried film of stain. Retard the drying of the blood by placing the slide in a covered dish or coplin jar which contains a moistened filter paper. Let stand for 10 minutes.

Remove slide from moist chamber. Let film of blood dry in air and counterstain with Wright's. Count the reticulocytes.

The advantage of this method is that counts can be done at a later date, whereas in the other methods the counts must be made within a

few hours. It is advisable to make more than one preparation and to use great care in preventing drying of the blood smear before the dye has had time to penetrate the red cells and stain the granulofilamentous structures.

Clinical Applications

The normal values for reticulocytes is usually within the range of 0.1 to 2 per cent, with occasional normal values as high as 4 per cent. An increase in the number of reticulocytes in the peripheral blood is indicative of erythropoietic hyperactivity while a decreased number or a normal or only slightly increased number in the presence of anemia is indicative of hypoactivity or inadequate regenerative response. In severe aplastic states the reticulocytes may be difficult to find, whereas in regenerative diseases such as sickle cell anemia or spherocytic anemia, the reticulocytes may be above 25 per cent. For reticulocyte responses in the various blood dyscrasias see Chapters 3 and 5.

In diseases in which there is defective maturation and delivery of cells due to a deficiency of a specific substance such as the anti-pernicious anemia factor, the giving of the specific substance causes the immature red cells to mature. As millions of early erythrocytes simultaneously pass through the various stages of development from rubriblasts to metarubricytes and finally to the diffusely basophilic red cell stage, there comes a time when the number of immature cells (reticulocytes) is greatly increased. In pernicious anemia patients, given adequate doses of liver extract, B_{12} or its equivalent, this reticulocyte shower usually begins after the second to fourth day and reaches a peak within 5 to 10 days (Fig. 1.51), following which the reticulocyte count returns to normal. The height of the reticulocyte rise is usually proportional to the severity of the anemia provided an adequate dose has been given. The reticulocyte increase may be seen in iron deficiency anemia following iron administration, in thyroid deficiency states after thyroid extract and in anemias associated with vitamin deficiencies, after the specific vitamin is given.

The reticulocyte count is used in the bioassay of drugs and in testing the relative potency of several drugs. If one drug such as a certain iron preparation is given to a patient with iron deficiency anemia, producing a slight reticulocyte increase and is followed by a second iron preparation which produces a second reticulocyte shower, the assumption is that the second drug was more potent in dosages used than the first or that it supplied another deficient factor.

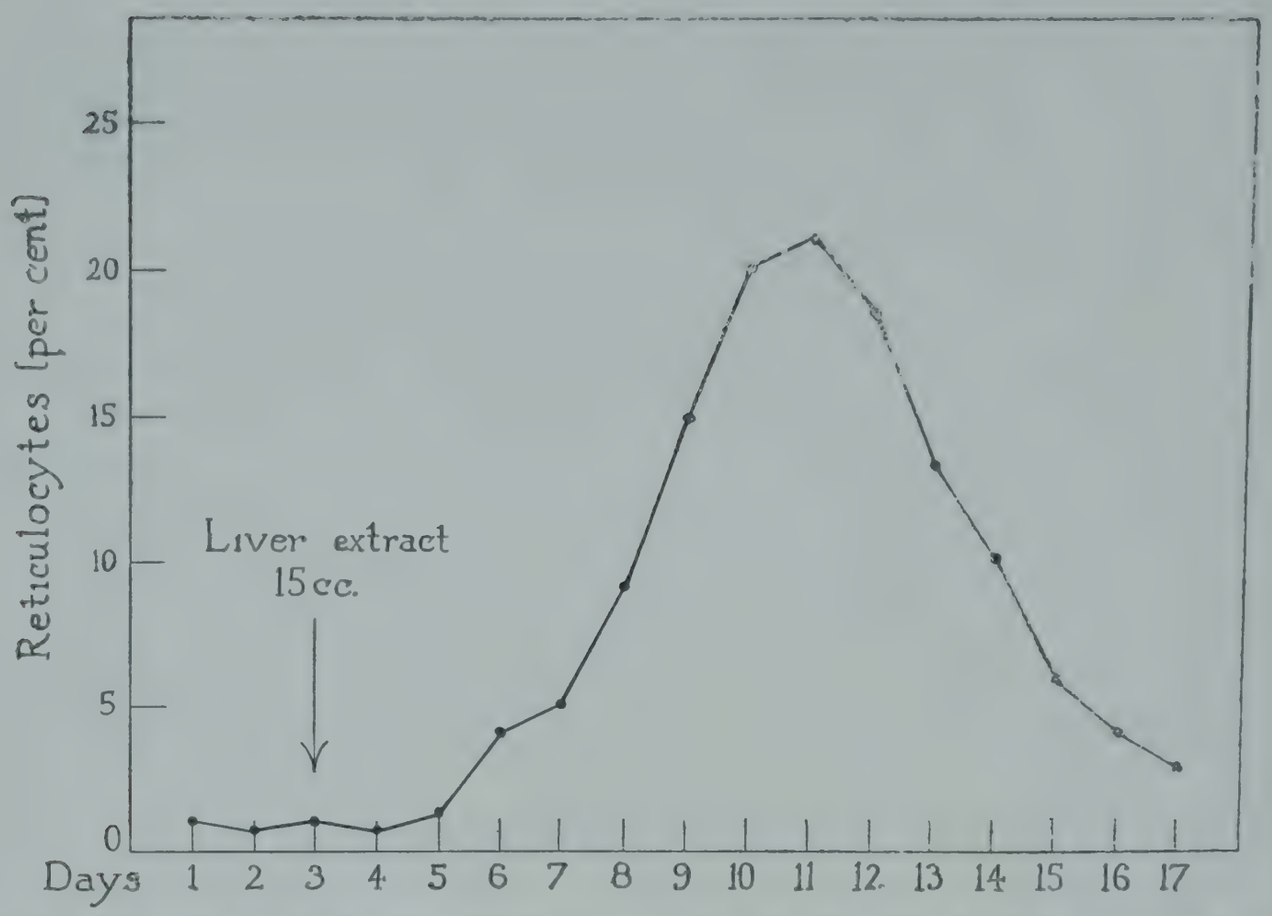


FIG. 1.51. Characteristic reticulocyte response in a patient with untreated pernicious (Addisonian) anemia, after a single intramuscular injection of concentrated liver extract.

OSMOTIC FRAGILITY TEST

When normal red cells are placed in hypotonic salt solutions, the cells absorb fluid causing the volume to increase and the shape to change from that of biconcave discs to spherical forms. Further expansion of volume leads to cell rupture or hemolysis. Cells which are thicker than normal or spherical cannot expand as much as can normal cells without placing their capsule under tension and therefore rupture more readily in low salt concentrations than do normal cells. On the other hand, cells which are thin (leptocytes), sickled cells or cells with surface folds (target cells), are capable of a greater than normal percentile expansion of volume before reaching the spherical shape. These thin cells therefore do not hemolize in hypotonic salt solutions as readily as do normal cells.

In testing the osmotic fragility of erythrocytes, blood is placed in a series of tubes containing descending concentrations of sodium chloride. The red cells are allowed to remain for several hours and the point of beginning and of complete hemolysis noted. Control tests are always needed for there are many variables such as the criteria used in evaluating hemolysis, the accuracy of measurements of the salt concentrations, the chemical purity of the salt used, the way the glassware is cleaned

the amount of blood in relation to the anticoagulant, and the time elapsed between the drawing of blood and the reading of the test.

For research purposes, methods are employed which involve preliminary washing of cells in physiological saline, uniform concentrations of cells and quantitative measurement of the hemolysis by determination of the amount of hemoglobin in the supernatant fluid or by measuring the cell volume or counting the red cells remaining.

Methods

Qualitative.

A stock solution of sodium chloride is prepared by dissolving 1 gm. of "C.P." sodium chloride, which has been dried in a dessicator, in 100 ml. of distilled water. By using varying amounts of this 1 per cent salt solution and making up to 10 ml. with distilled water, the desired ranges of salt concentration are prepared. Thus, if a 0.50 per cent solution is desired, 5.0 ml. of stock solution is mixed with 5 ml. of water; for a 0.48 per cent solution, 4.8 ml. of stock solution plus 5.2 ml. of water; etc.

As a screening procedure to detect the range of salt solutions required for a given case, solutions having a salt concentration of 0.85 per cent and 0.50 per cent can be set up. One drop of venous or oxalated blood is added to 10 ml. mixed and centrifuged. If hemolysis occurs in the 0.50 per cent tube, solutions above this level must be included in the test, if there is no hemolysis in this tube, the testing range is below this level.

The usual range is provided by making up a duplicate series of 12 tubes, each set having concentrations 0.50 to 0.30 per cent at intervals of 0.02 per cent.

After venous blood is collected, the needle is removed and one drop of blood is added to each tube. If the patient is anemic, two drops are preferred. The blood first may be mixed with ammonium and potassium oxalate and 0.1 ml. or 1 drop from a 1 ml. pipette added to each tube. The blood from the control and the patient must be handled in exactly the same way. After thoroughly mixing, the tubes are allowed to stand for several hours preferably in a refrigerator until the red cells settle. If more rapid reading is desired, the tubes may be centrifuged.

The point of beginning hemolysis in each series is noted by looking for the tube with the highest concentration in which the pink tinting of the supernatant fluid is detectable. A bright light, a white background, and comparison with other tubes help in determining this end point.

The point of complete hemolysis is taken as the tube in which there

are no red cells left intact. Aids in determining this end point are a slight agitation of the sediment to see if whorls of cells come up from the bottom, or reading print through the solutions as a means of detecting turbidity. The solution at the point of complete hemolysis is clear.

Example of Report:

	Beginning Hemolysis	Complete Hemolytis
Patient	0.48% NaCl	0.36% NaCl
Control	0.44% NaCl	0.30% NaCl

Quantitative.

1. Single tube screening test. Because of the increased interest in hemolytic diseases and the frequent requests for time-consuming osmotic fragility studies, it is recommended that a preliminary quantitative photoelectric measurement of the degree of hemolysis in one concentration of saline (0.5 per cent NaCl) be performed.

The skin is punctured and 0.02 ml. of capillary blood mixed with 5 ml. of dilute NH_4OH. The concentration of hemoglobin in this completely hemolyzed specimen is determined by the oxyhemoglobin method. This concentration serves as a standard or 100 per cent. In another tube 0.02 ml. of capillary blood is mixed with 5 ml. of a 0.5 per cent NaCl solution (dilution 1:250). This second tube is centrifuged to remove the unhemolyzed cells. The concentration of hemoglobin in the supernatant fluid is determined photometrically. The reading should be made within two hours. The degree of hemolysis is calculated:

$$\frac{\text{Hemoglobin Reading of Saline Supernatant}}{\text{Hemoglobin Reading of } NH_4OH \text{ Solution}} 100 = \% \text{ Hemolysis}$$

In the majority of individuals there is a range of variation from 0 to 30 per cent with the mean hemolysis 10 per cent. In patients with hemolytic anemia the hemolysis per cent is 30 per cent or more. If the degree of hemolysis in the 0.5 per cent saline tube is less than 25 per cent, it is unnecessary to further investigate the patients red cell fragility. If there is more than 40 per cent hemolysis, a significant increase in osmotic fragility is established (Enticknap, J. B.: Guy's Hosp. Rep. 101: 64–67, 1952).

2. Multiple tube hemolysis test. Procedures involving the estimation of the degree of hemolysis in various concentrations of saline solution are needed in research studies of hemolytic processes and in evaluating minor degree of change, but are too time-consuming to be used in practice (Shen, S. C., Ham, T. H. and Fleming, E. M.: New England J. M. 229: 701, 1943).

Clinical Applications

When normal blood is mixed with descending concentrations of hypotonic salt solution, some of the red cells undergo hemolysis within the range of 0.48 to 0.40 per cent NaCl (average 0.44 per cent) and there is complete hemolysis between 0.36 and 0.28 per cent NaCl (average 0.34 per cent).

In hereditary spherocytic anemia, and in certain patients with acquired hemolytic jaundice, there is partial hemolysis in concentrations above the control and often above 0.50 per cent NaCl. In thalassemia, sicklemia and obstructive (regurgitant) jaundice, hemolysis occurs at levels lower than the control and there is often a greater spread than normal between the tubes in which there is beginning hemolysis and complete hemolysis. In sicklemia and thalassemia, and perhaps in other conditions, it is thought that the red cells are not only thinner than normal, and therefore can expand to a greater extent before hemolysis occurs, but that these cells act as poor osometers and do not imbibe fluids as readily as do normal cells.

The fragility test is a fairly time-consuming procedure which is not indicated unless hemolytic jaundice is suspected. Indications for ordering the test are unexplained jaundice of a hemolytic type, abnormal red cell regeneration and splenomegaly. The test is not needed in the diagnosis of sickle cell anemia or in obstructive jaundice. In the majority of cases of anemia in which there is no evidence of abnormal blood destruction, nor compensatory regenerative signs, the test should not be ordered for it will be of no value and will add pain, expense and unnecessary labor.

CHOICE OF PROCEDURES

The hematological procedures which are most useful as screening tests, arranged in the order of preference are:

1. Blood smear examination
2. Hematocrit or hemoglobin
3. Sedimentation rate
4. White cell count.

The most simple combination of screening tests and one which can be made in the patient's home is the blood smear and the hemoglobin determination. The number of white cells can be roughly estimated by the examination of the thin end of the blood smear. In normal blood the number of leukocytes per low power field varies between 5 and 20, whereas in significant leukocytoses there are more than 20 and

in neutropenic states there are less than 5. The smear examination should not be limited to the differential white cell count, but should include an appraisal of the size, shape and color of the red cells and the number of thrombocytes.

For office or clinic patients presenting diagnostic problems and for hospital routines, the recommended combination of procedures includes the blood smear, sedimentation rate, hematocrit and serological test for syphilis. The examination of the blood smear and the measurement of the buffy coat at the top of the red cell column in the hematocrit will detect significant abnormalities in the white cell count, but if desired, the white cell count may be included in the list of screening tests. The recommended procedure is to perform a single venipuncture using a 20-ml. syringe and a 20 gauge needle and to collect approximately 15 ml. of blood. A small drop of blood is placed on the end of several slides and thin, margin free spreads are made. The needle is then removed from the syringe, 5 ml. of blood placed in a bottle containing dried ammonium and potassium oxalate and the blood and anticoagulant thoroughly mixed (see under anticoagulants). The remaining blood is placed in a dry serological tube and allowed to clot. One milliliter of oxalated blood is placed in a Wintrobe tube for the sedimentation rate and the packed cell volume. The remaining oxalated blood can be used for hemoglobin concentration, cell counts or numerous other hematological or chemical procedures if desired. The clotted blood is used for the serological test for syphilis but can also be used for typing and cross matching, bilirubin estimation, or agglutinations, heterophile antibody determinations or other bacteriological procedures.

The technician performing the venipuncture has to be skilled and has to work rapidly in order to make satisfactory blood smears from the venipuncture needle and to get the blood into the oxalate bottle before blood begins to clot. If there is difficulty in venipuncture or delay in drawing the blood, the blood smear should be made from a finger puncture wound and not from the venipuncture needle. Blood smears made from fresh oxalated blood are suitable for differentiating the main cell types and are therefore acceptable as screening procedures, but are not to be relied upon for the diagnosis or differential diagnosis of blood dyscrasias.

There is little justification for performing more than one routine test in order to detect anemia. Of the three tests in common use, the hematocrit is the most simple and most reliable and the hemoblogin concentration the second choice.

The performance of the red cell count on all patients in addition to

the hematocrit or hemoglobin estimation is superfluous and unreasonable, for the red cell count takes much time and has a large margin of error. It would be much more advantageous to perform a few red cell counts on selected patients who have anemia or need diagnostic studies, than it would be to perform hurried and unreliable red cell counts on everyone.

The physician making home calls should be prepared to make blood smears at the bed side, to perform a Tallqvist hemoglobin and to collect venous blood for hematological, serological, and chemical tests. The equipment in the medicine bag should include:

1. Sterile cotton or gauze.
2. Hemolet.
3. New, clean slides in a covered box or wrapped in paper.
4. Tourniquet.
5. Syringe and needles.
6. Bottles or tubes containing ammonium and potassium oxalate with screw tops or stoppers.
7. Sterile serological tubes, with stoppers.
8. Pocket size, book type of Tallqvist hemoglobin scale.

If the initial laboratory procedures reveal anemia or abnormalities in the white cells, the following diagnostic study should be made:

Hemoglobin concentration
Red cell count
Hematocrit
Calculation of the mean corpuscular values
Reticulocyte count
White cell count

If there is suspicion of increased blood destruction as revealed by clinical jaundice, increased pigment in the supernatant fluid in the hematocrit tube or a regenerative blood picture, the following tests should be added to the above diagnostic study:

Serum bilirubin determination
Tests for bile pigments in the urine
Erythrocyte osmotic fragility

If there are petechiae, ecchymoses or spontaneous bleeding from mucous surfaces, the following screening hemorrhagic studies should be performed:

Bleeding time, Ivy method, which includes a capillary fragility test
Coagulation time, multiple tube method with examination of the clot for retraction, firmness, etc.
Prothrombin concentration

For other tests indicated in specific diseases, see Chapters 3, 4, 5, and 6.

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CHAPTER 2

BLOOD CELLS. BONE MARROW EXAMINATION

By L. W. Diggs

FORMATION OF BLOOD CELLS

In the embryo the first recognizable blood cells arise in the mesenchymal layer from fixed reticulum cells and from the lining (endothelial) cells of the primitive blood vessels. In the early months of fetal life the principal sites of blood formation are in the liver and spleen, but as the bone marrow develops, the formation of blood cells is more and more taken over by this tissue.

In fetal life and in infancy the marrow is mostly of a cellular type and there is little fat, but in later childhood the cellular or red marrow recedes from the more distal bones and the major formation of blood cells takes place in the flat or "warm" bones of the body, such as the sternum, ribs, vertebrae, and bones of the pelvis, shoulder girdle and skull. In the adult there is little actively regenerating marrow in the long bones except at the upper ends of the humerus and femur. There remain, however, in the marrow cavity of long bones stem cells which are capable of reproducing blood cells when there is need and proper stimulation.

In the adult the majority of new cells are formed by mitoses of cells that are already differentiated, producing cells of like morphology (homoplastic division). Thus an eosinophilic myelocyte divides and produces two eosinophilic myelocytes, which from that point on may mature without further division or may redivide at the same level or partially mature and redivide at a more mature level. On the other hand, cells which have their origin in embryonal mesenchyme are multipotent and are capable of producing more than one strain of cells (heteroplastic division) under different environmental conditions.

Primitive cells are said to be differentiated when they develop distinctive structural characteristics, acquire functional capacities such as phagocytosis or are associated with protein synthesis and tissue changes of specific type. Thus an undifferentiated mesenchymal cell becomes a fibroblast when it forms or is associated with the formation of collagen fibers, a reticular cell when it develops reticular fibers which can be demonstrated by silver and other special stains, an osteoblast or a chondroblast when it is associated with bone or cartilage formation, a rubriblast when it forms hemoglobin, etc.

The most primitive cells are fixed tissue cells, which are firmly attached to each other. As the cells mature, the intercellular connecting substance disappears and the cells become free cells, which are mechanically swept into the peripheral blood from margins of growing colonies or get into the blood stream by means of their own motility. The majority of cells are formed extravascularly, but the erythrocytes are thought to be formed in the sinusoids of the marrow and the monocytes to be formed partially within blood vessels.

The stimuli for the formation of cells and the controlling factors that regulate the growth and delivery of cells from their fixed state into free cells are not known. Control by substances from various glands of internal secretion or by the central nervous system has never been clearly established. Anoxemia is one known stimulus for the formation of cells of all types.

The lineage of the various cell types and their relation to the primitive cells and to each other have been the subject of continual study for many years, but there is still lack of agreement as to terminology and the developmental potentialities and limitations of various stem cells. One scheme of classification and a nomenclature for the various maturation stages are summarized in Table 2.1. In this scheme it is to be noted that all cells arise from the undifferentiated stem cell and that the primitive cell of each cell type with the exception of the histiocyte is represented by a parent cell designated by the suffix "blast," meaning the seed or originating cell.

THE HEMATOPOIETIC ORGANS

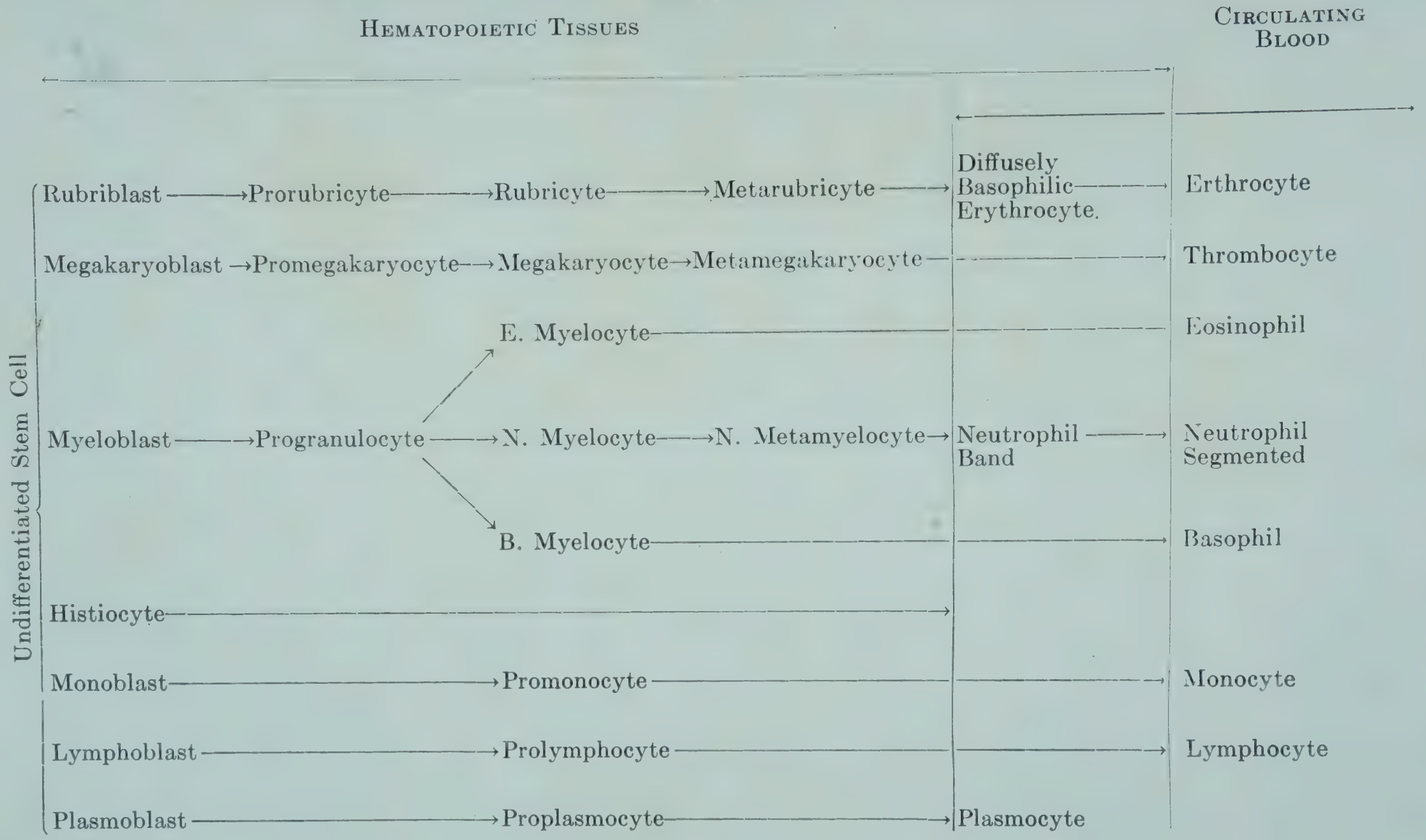
Bone Marrow

The bone marrow produces red cells, thrombocytes (platelets), neutrophils, eosinophils, and basophils, and serves as one of the sites of formation of lymphocytes, monocytes, histiocytes and plasmocytes.

The total volume of the bone marrow in all of the bones of the body has been estimated as greater than that of the liver.

The marrow within the spaces bounded by the bony trabeculae is composed of delicate supporting structure of reticular fibers, varying amounts of fat, and richly anastomosing vascular channels, many of which are supposedly collapsed at any given time. The mesenchymal cells which produce the blood cells and the marrow cells of different types and in different stages of maturation are intermingled in the tissue spaces.

TABLE 2.1


Hematopoietic Tissues
Circulating Blood
Undifferentiated Stem Cell
Rubriblast
Prorubricyte
Rubricyte
Metarubricyte
Diffusely Basophilic Erythrocyte.
Erthrocyte
Megakaryoblast
Promegakaryocyte
Megakaryocyte
Metamegakaryocyte
Thrombocyte
E. Myelocyte
Eosinophil
Myeloblast
Progranulocyte
N. Myelocyte
N. Metamyelocyte
Neutrophil Band
Neutrophil Segmented
B. Myelocyte
Basophil
Histiocyte
Monoblast
Promonocyte
Monocyte
Lymphoblast
Prolymphocyte
Lymphocyte
Plasmoblast
Proplasmocyte
Plasmocyte

The fat in the marrow occupies reserve space and readily goes into solution and disappears as the blood cells increase, thus making it possible for regeneration to occur on demand without interfering with vital structures.

Lymphatic System

The lymphocytes are formed for the most part in the lymph nodes and in the islands of lymphatic tissue which are located in the spleen, bone marrow, thymus, liver and intestinal tract and to a lesser extent in practically every other organ except the brain and spinal cord. In addition to organized masses of lymphocytes there are many scattered lymphocytes all over the body. The combined lymphatic tissue is estimated to constitute 0.5 per cent of the total body weight.

The lymphatic tissue is depressed and undergoes degenerative changes when stimulated by cortical hormones and tends to undergo hypertrophy when there is cortical hypoadrenalism.

Spleen

The spleen aids in the formation of lymphocytes and monocytes and serves as a reservoir for cells which can be delivered from the pulp into the circulation on demand. The spleen through its filtering and macrophage system is important in defense against infections and in antibody production.

Red cells, leukocytes and platelets are destroyed in large numbers in the spleen, for the cells in the extensive vascular channels and splenic cords are in intimate contact with phagocytes. The organ is thought to have an inhibiting effect on the bone marrow cellular activity, perhaps by means of a circulating hormone.

Liver

The liver, through its fixed macrophages, the Kupffer cells, aids in blood destruction. The large vascular channels which are capable of constriction and dilatation aid in the regulation of blood volume. The liver also is the excretory organ for bile pigment.

Reticuloendothelial System

The reticuloendothelial system is part of a system of macrophages which are capable of phagocytizing particulate matter and storing colloidal substances (Aschoff) and in a broader sense as defined by Klemperer "a system of cells widely distributed throughout the organism which have their origin in embryonal mesenchyme and retain the po-

tentialities of embryonal mesenchyme throughout the postnatal life of the organism." This cell system in the adult organism is principally concerned with the formation of histiocytes and related phagocytic or potentially phagocytic cells, but is also the potential source of all other mesenchymal elements including the blood cells.

Other Organs

It is thought that some of the megakaryocytes and portions of the megakaryocytes escape from the bone marrow and lodge in the lung capillaries, from which site they continue to produce thrombocytes.

In malignancies involving the blood cells, the skin, kidneys and other organs become infiltrated with abnormal blood cells and produce their type in these metastatic foci.

FUNCTION OF BLOOD CELLS

The blood cells have various functions. The red cells serve as transporters of oxygen and carbon dioxide, and aid in maintenance of acid-base equilibrium. The neutrophils, monocytes, histiocytes and to a lesser extent the eosinophils have the power of phagocytosis of bacteria and particulate matter and the monocytes and histiocytes (macrophages) have the power of phagocytizing larger particles, such as whole cells, nuclear fragments or protozoa. Proteolytic enzymes, lipase and other enzymes have been demonstrated in leukocytes. The lymphocytes, plasmocytes, monocytes and histiocytes are intimately concerned with the manufacture of antibodies. To the eosinophils has been ascribed the property of detoxification. The function of basophils has not been determined. The function of the white cell in general may be said to be that of maintaining the defenses of the body against invasion by microscopic organisms and in removing dead cells and foreign material.

Thrombocytes (platelets) aid in stopping blood flow from injured blood vessels by forming aggregates or platelet thrombi which mechanically plug the ends or holes in blood vessels and aid in closing the wound. On disintegration the platelets liberate activator substances which convert plasma thromboplastinogen to active thromboplastin and constrictor substances which aid in blood vessel lumen closure and retraction. Thrombocytes also play a role in the retraction of the blood clot and aid in the localization of bacteria and other small objects by adhering to these objects and producing aggregates too large to pass through capillsries.

FATE OF BLOOD CELLS

As the cells circulate through the vascular channels, they are continually losing cytoplasmic substance to the plasma. Whole cells or fragments may be phagocytized. Phagocytosis takes place for the most part in the reticular-lined spaces of the spleen, but to a lesser extent in the specialized endothelium of the bone marrow, lymph nodes and liver. When cells or cell constituents are taken into the cytoplasm of other cells, they are digested, the usable food factors made available for other cells, and the non-usable factors passed back into the blood stream to be excreted by the liver and kidney.

The cells thought to have the longest expectancy of life are the histiocytes, which may survive for years. Red cells remain intact for 80 to 120 days. Myeloid cells, lymphocytes and monocytes live for several days or weeks after they escape into the general circulation. Platelets are the most fragile of all blood structures, their average life span in the circulation being a matter of days. Some of the cells are being continually lost from the blood vessels as evidenced by the fact that leukocytes are found in all serous fluids, in perivascular tissues and in secretions of the gastro-intestinal, genito-urinary and respiratory tracts.

The number of cells present in the body at any given time is determined by the balance between the rate of blood formation and loss and destruction. Many of the capillaries and sinusoids of the vascular system contain mature cells which are not actively circulating. These cells may be mobilized in case of demand.

NOMENCLATURE

The nomenclature of cells used is that recommended by the "Committee for the Clarification of Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs," sponsored by the American Society of Clinical Pathologists and the American Medical Association, published in the American Journal of Clinical Pathology (18: 443–450, 1948; 19: 50–60, 1949; and 20: 562–579, 1950).

In this terminology the most undifferentiated cell of each series carries the suffix "blast," and the second stage, the prefix "pro." All cells more mature than the blast stage have names which are specific for the series, followed by the suffix "cyte." The name of the fourth stage of cells of the granulocytic and erythrocytic series has the prefix "meta." Examples are given in Table 2.2.

The most radical changes recommended involve the cells of the erythrocytic series. It was considered impossible to reconcile the various

TABLE 2.2

Prefix	Suffix	Granulocytic (Myelocytic) Series	Erythrocytic (Rubricytic) Series	Lymphocytic Series
	——blast	*myelo*blast	*rubri*blast	*lympho*blast
pro——	——cyte	pro*granulo*cyte	pro*rubri*cyte	pro*lympho*cyte
	——cyte	*myelo*cyte	*rubri*cyte	*lympho*cyte
meta——	——cyte	meta*myelo*cyte	meta*rubri*cyte	

terminologies used in the past for the various stages of the nucleated red cells; the same terms had multiple meanings, the suffix "blast" was used for nucleated red cells of all stages of development and there was unfortunate use of separate terms for the dysplastic large cells found in pernicious anemia (megaloblasts) and the normal cells (normoblasts). The most commonly used synonyms for the terms rubriblast, prorubricyte, rubricyte and metarubricyte are given in Table 2.3.

It is to be expected that many physicians will continue to use the terms to which they have been accustomed and that some will vigorously oppose any attempt to change or to standardize terminology. For medical students who are learning for the first time the names of cells and who do not have the historical background for the understanding of many of the terms formerly used, the new terminology has proved to be satisfactory and an improvement over the confused terminology of the past.

CELL MORPHOLOGY

General

All types of cells reveal comparable changes as they develop from immature or primitive cells to mature definitive types (Plates 2.I and 2.II).

Early cells as a class are larger than the mature cells. The nucleus of early cells as a class are larger than the mature cells. The nucleus of early cells is larger than that of mature cells and is relatively larger in relation to the cytoplasm.

The cytoplasm of immature cells stained by eosin and polychrome methylene blue as in Wright's stain takes a blue color. As the cells mature, the cytoplasm stains less blue and more red. Granules are not present in primitive undifferentiated cells but develop as the cells differentiate. As a rule, the first granules to appear stain blue or dark shades of purple, intermediate granules are more red and highly developed and differentiated granules are bright red.

The nuclei of primitive cells are round. As the cells develop, the

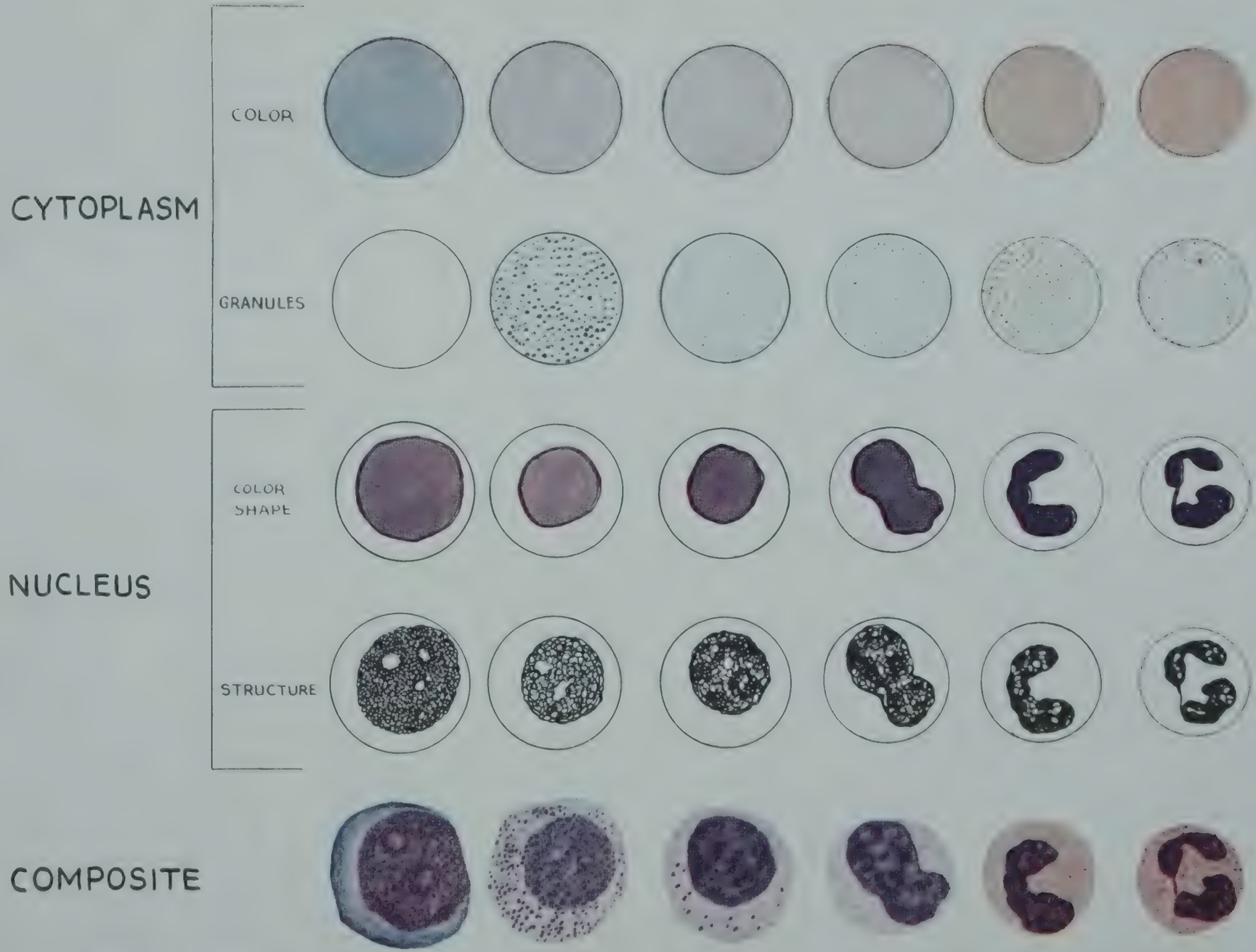


PLATE 2.I. Changes in cell characteristics as they mature. The most immature forms are on the left and the mature forms on the right.

Bottom line: Maturation sequence of neutrophils (Wright's Stain). Left to right: Myeloblast, progranulocyte, myelocyte, metamyelocyte, band, segmented.

TABLE 2.3

TERMS IN COMMON USE FOR NUCLEATED RED BLOOD CELLS

rubriblast	proerythroblast		pronormoblast		megaloblast
prorubricyte	early basophilic	erythroblast	early basophilic	normoblast	early erythroblast
rubricyte	intermediate polychromatic	erythroblast	intermediate polychromatic	normoblast	late erythroblast
metarubricyte	late orthochromatic acidophilic oxyphilic eosinophilic	erythroblast	late orthochromatic acidophilic oxyphilic eosinophilic	normoblast	normoblast

nuclei become indented, lobulated or fragmented. The color of the stained nucleus of early cells is red to purple, whereas the color of mature nuclei is predominantly blue. In early cells the nuclei may be lighter than cytoplasm, whereas in mature cells the reverse is true. The chromatin structure of primitive cells is very fine and lace-like, in intermediate cells it is more coarse and ropy and in older cells it is matted together in masses. Nucleoli are present in stem cells, become indistinct in later forms and are absent in mature cells.

Mitotic figures and irregular cytoplasmic tags extending from the margins of the cells are indicative of immaturity, whereas pseudopods and phagocytized material in the cytoplasm are indicative of maturity.

The cytoplasmic, granular and nuclear changes do not always take place at the same rate of speed. Primitive cells that are rapidly dividing may be quite small and cells lacking essential maturation factors may grow without dividing and become abnormally large. Maturation sequences that are out of step with each other are indicative of abnormality.

In any given cell sequence there are cells which are morphologically borderline between one stage of development and the next. In naming such cells the rule is "to bet on the favorite instead of the dark horse" or to give the benefit of the doubt to the most frequently occurring variant which is usually the more mature form.

When cells do not fall into well defined classifications but closely resemble each other, it is recommended that a new heading or column be provided for the purposes of tallying. When these atypical cells are reported, the morphological features of the group are described and the number answering this description stated.

Cells of the same type do not always look exactly alike. No description or picture of a single cell can possibly portray all of the morphological variations any more than one pin-up girl can represent all of the variants of female pulchritude.

Stem Cells

In attempting to classify various cells of the bone marrow or other body fluids, one encounters primitive cells which have no identifying features to indicate the specific cell family to which they belong or the type of cells which they are destined to beget. For such undifferentiated mesenchymal cells the category or column labelled "stem cells" is provided. Commonly used synonyms for stem cells are "reticular cells," "reticulo-endothelial cells," "hemohistioblasts," and "polyblasts."

These primitive cells have the morphological features common to all "blasts," namely a round and relatively large nucleus with a finely reticulated nuclear chromatin which takes a reddish stain, well defined nucleoli and a bluish non-granular cytoplasm. The intensity of the basophilia varies widely. As a rule the cytoplasm has a mottled or foamy structure, and is likely to be less deeply stained near the nucleus than at the periphery. Stem cells may be very large (25 to 40 microns) or as small as small lymphocytes (10 to 15 microns) depending on the frequency of mitoses and the period of growth between cell divisions. Because these cells are fixed tissue cells which are torn away from their cytoplasmic connections when the material is collected, their margins are often torn or jagged or there are blunt protoplasmic projections due to partial rounding up (Plates 2.II and 2.IV).

If primitive cells are associated with large numbers of cells which can be identified or are surrounded by differentiated cells, the specific family prefix is used with the term "blast" rather than the non-specific term "stem cell." Thus a stem cell in a bone marrow smear from a patient with lymphocytic leukemia is called a "lymphoblast," a primitive cell from a plasma cell tumor is called a "plasmoblast" and an undifferentiated cell in a marrow characterized by marked erythrocytic hyperplasia is probably a "rubriblast" (proerythroblast).

Erythrocytic Series, Normal Sequence

Rubriblast (proerythroblast, pronormoblast, megaloblast (Sabin)). Rubriblasts have the characteristics of other "blast" forms. In the earliest forms the cytoplasm stains light blue, but in later and more frequently occurring forms, presumably in association with beginning hemoglobin formation, there is a superimposed reddish tint which imparts to the cytoplasm a peculiar dark purplish royal blue color similar to that seen in plasma cells (Plates 2.II and 2.III and Fig. 2.1). Rubriblasts constitute less than 1 per cent of the nucleated red cells of normal marrow.

Prorubricyte (basophilic erythroblast, basophilic normoblast, early erythroblast). This cell is slightly smaller than the rubriblast and the nuclear structure is less delicate. The nucleoli are indefinite. The cytoplasm is still predominantly blue or basophilic (Plates 2.II and 2.III and Fig. 2.1). These cells constitute 1 to 4 per cent of the nucleated marrow cells.

Rubricyte (polychromatophilic erythroblast or normoblast, intermedi-

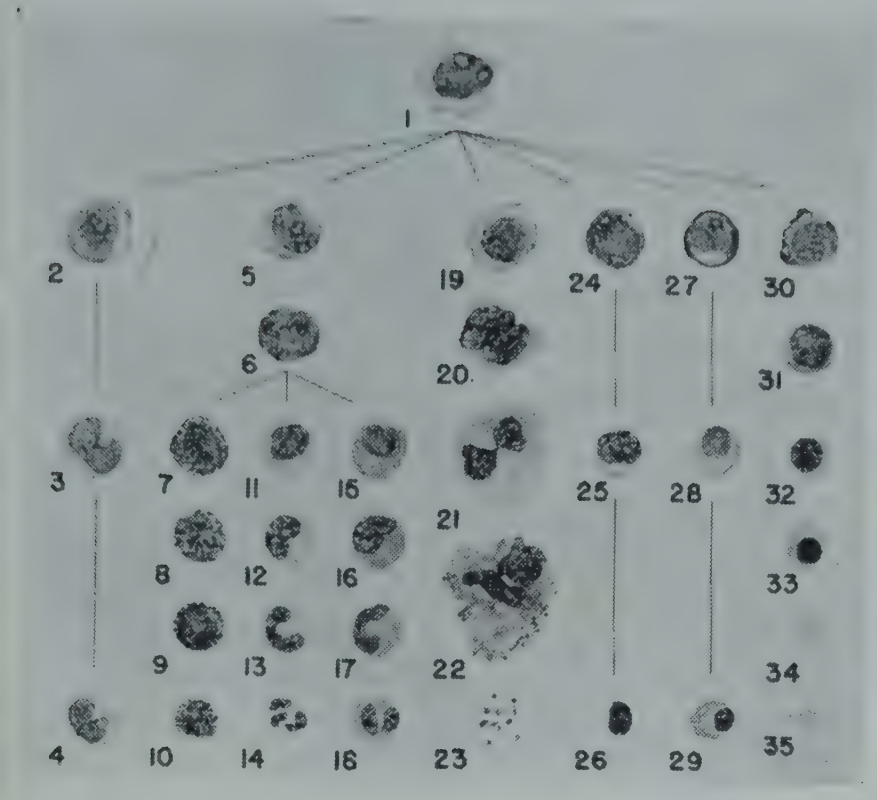
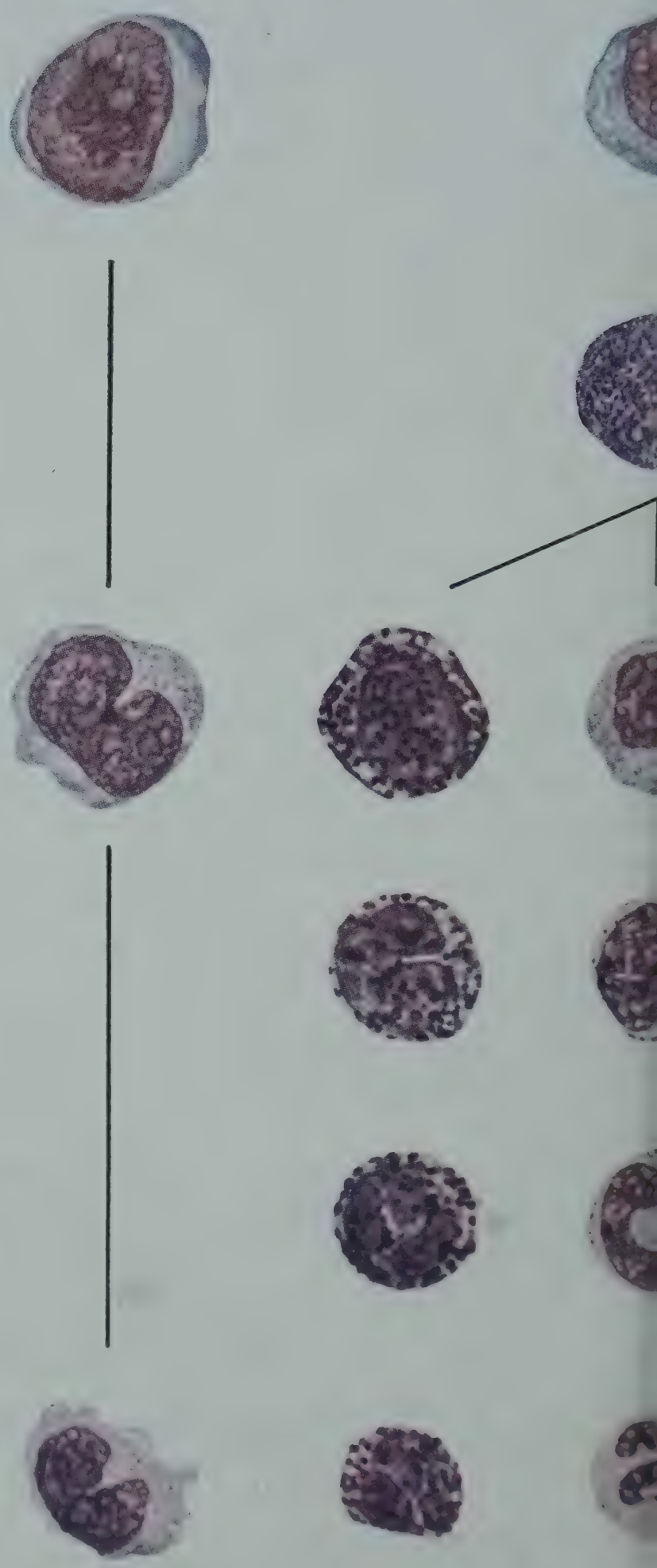
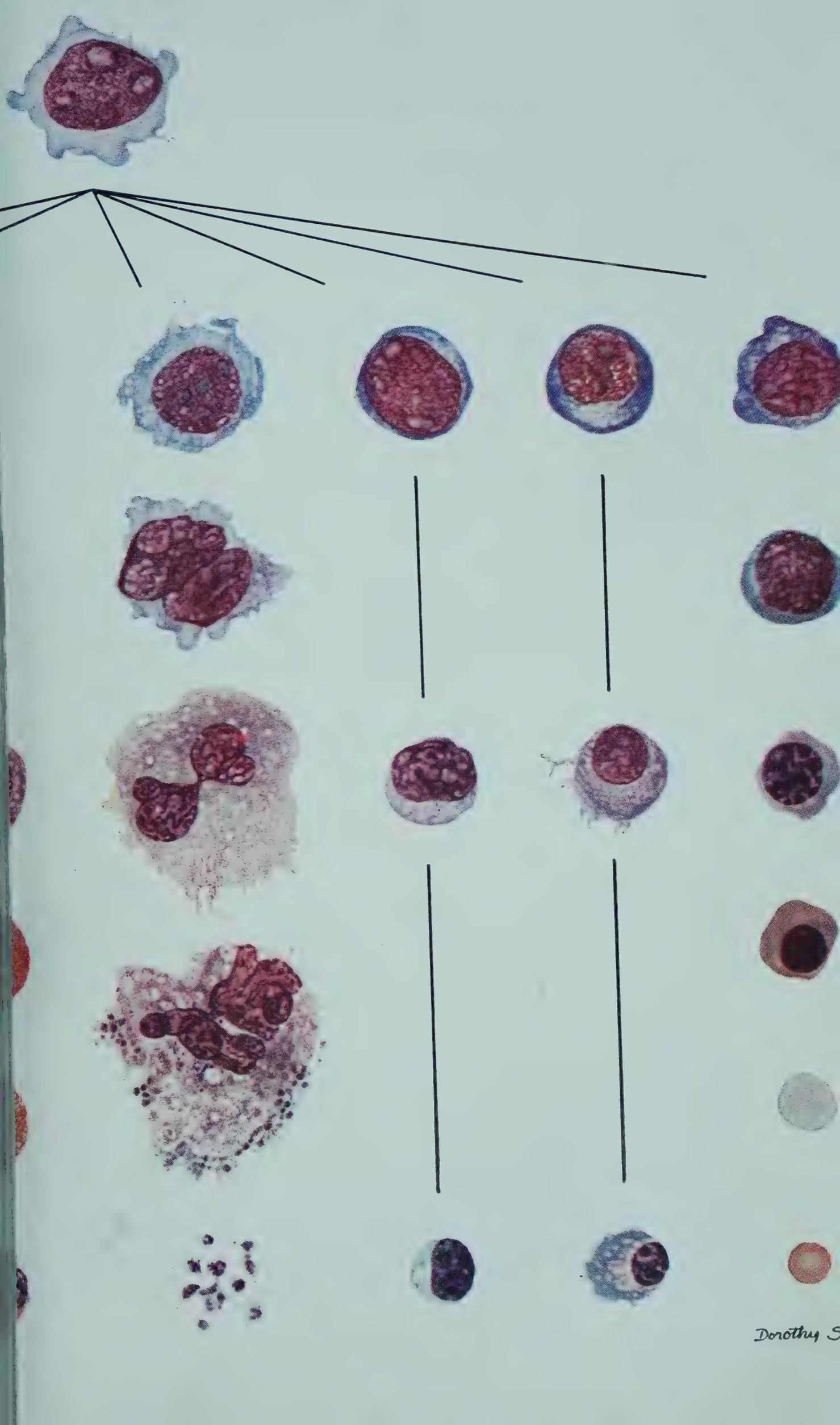


PLATE 2.II

Origin and Development of Blood Cells

1. Stem cell
2. Monoblast
3. Promonocyte
4. Monocyte
5. Myeloblast
6. Progranulocyte
7. Basophilic myelocyte
8. Basophilic metamyelocyte
9. Basophilic band
10. Basophilic segmented
11. Neutrophilic myelocyte
12. Neutrophilic metamyelocyte
13. Neutrophilic band
14. Neutrophilic segmented
15. Eosinophilic myelocyte
16. Eosinophilic metamyelocyte
    (See note to 18 below.)
17. Eosinophilic band
18. Eosinophilic segmented
    (NOTE: In key plate above, No. 18 is in bottom row, incorrectly numbered 16.)
19. Megakaryoblast
20. Promegakaryocyte
21. Megakaryocyte without thrombocytes
22. Metamegakaryocyte
23. Thrombocytes
24. Lymphoblast
25. Prolymphocyte
26. Lymphocyte
27. Plasmoblast
28. Proplasmocyte
29. Plasmocyte
20. Rubriblast
31. Prorubricyte
32. Rubricyte
33. Metarubricyte
34. Diffusely basophilic erythrocyte
35. Erythrocyte





ate erythroblast or normoblast). The nucleus of the rubricyte is partially degenerate and resembles that seen in the mature lymphocyte. Nucleoli are no longer discernible. The cytoplasm is bluish red or polychromatic (Plates 2.II and 2.III and Fig. 2.1). The normal range of rubricytes in marrow is 5 to 10 per cent.

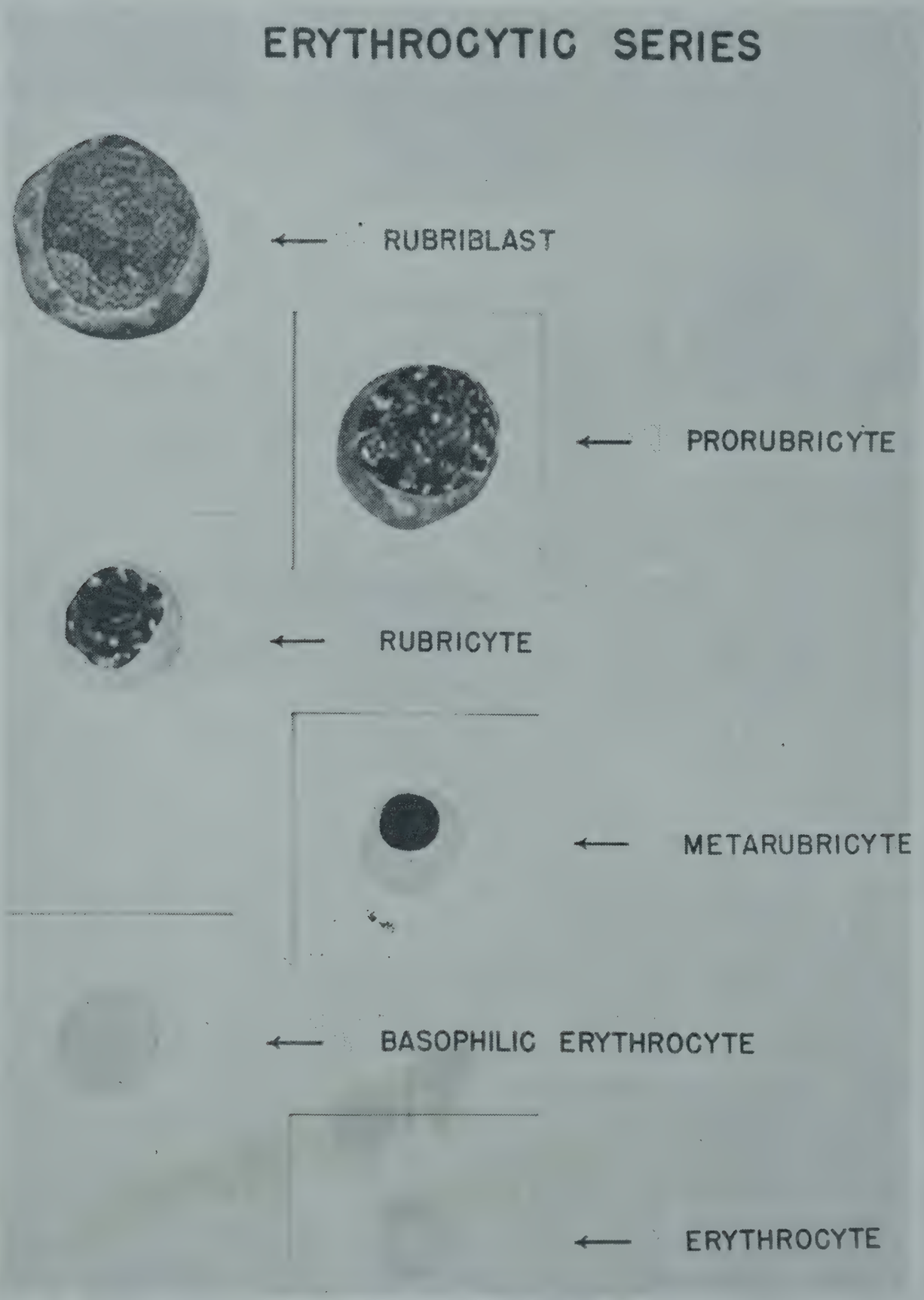


Fig. 2.1. Erythrocytic series

Metarubricytes (normoblast, orthochromatic erythroblast or normoblast, late erythroblast or normoblast, acidophilic, oxyphilic or eosinophilic erythroblast or normoblast). In this cell the cytoplasm is predominantly red, but as a rule there is slight residual blueness or basophilia. The amount of blue varies in individual cells. In some cells the cytoplasm stains in much the same manner as the normal nonnucleated red cells in the same fields, thus forming the basis for the prefixes "normo" and "ortho" which have been applied to these cells. The nucleus of metarubricytes is pyknotic and in its most characteristic form appears as a solid almost blue-black sphere (Plates 2.II and 2.III and Fig. 2.1).

The metarubricyte constitutes the most common form of nucleated red cells in bone marrow; the range of normal values is from 10 to 20 per cent. Metarubricytes like other nucleated cells of the red cell series are not found in the peripheral blood of normal individuals.

Diffusely Basophilic Erythrocytes (polychromatophilic erythrocytes). Cells of this type have lost their nucleus, but still stain blue. As a class, they are larger than normal and do not have central pallor. Cells which appear as diffusely basophilic cells in the Wright's stain would, if stained by brilliant cresyl blue or other supravital stains, reveal reticular structures. The number of diffusely basophilic cells in the peripheral blood of normal individuals is approximately 1 per cent, but in cases of abnormal regeneration, the number is increased (Plates 2.II and 2.III and Fig. 2.1).

Erythrocytes (normocytes, red blood cells). The mature erythrocytes are 6 to 8 microns in diameter. They stain a buff or reddish color. Their shape is that of a biconcave disc, which causes the cells to appear lighter in the center than at the periphery. Some of the cells may have cup or spherical shapes. Crenation and slight irregularities of the surface are considered as normal variants.

Abnormalities in Erythrocytic Cells

Macrocytic (Megalocytic) Cells of the Type seen in Pernicious Anemia and Related Anemias. In untreated pernicious anemia and in related macrocytic anemias due to deficiency of liver principle, the developing erythrocytes are abnormal and have morphological characteristics which are different from those found in normal marrow. The cells in all stages of development are larger than normal, which is due probably to the fact that the cells grow for longer periods of time without dividing, and that delivery into the circulating blood is delayed. There is evi-

dence of abnormal mitosis and of occasional cells with multiple nuclei. Early cells tend to have more cytoplasm in relation to nucleus. The cytoplasm is less homogeneous. Hemoglobin development and acidophilic cytoplasm are frequently found in cells with immature nuclei. Some hematologists claim to be able to distinguish the early cells of pernicious anemia from the young nucleated red cells of the normal marrow by minor differences in chromatin structure and nucleoli, but these differences are relative and non-specific.

Because many of the earlier studies of immature erythrocytes were performed on patients with pernicious anemia and because the red cells in this disease are so strikingly abnormal, special terminologies have been used for the cells found in this disease, the most acceptable of which are promegaloblasts, basophilic, polychromatophilic and orthochromatic megaloblasts and megalocytes. There is, however, no excuse for including the abnormal cells found in this disease in any classification of normal cells. There is no more reason for having a special terminology for the red cells of this disease than for the neutrophils or megakaryocytes in pernicious anemia which also tend to be larger and to have abnormal nuclear characteristics.

Microcytic Cells of the Type Seen in Iron Deficiency Anemia. In iron deficiency states due to any cause, the nucleated red cells at all stages of development are smaller than normal, their cytoplasm is bluer and contains less hemoglobin than do normal cells and the margins are often frayed and irregular. These cells fortunately have not received special names as have the dysplastic cells of pernicious anemia. In reporting such cells in a differential count a description should be given.

Nuclear Fragments. The nucleus of the nucleated red cell disappears after the metarubricyte stage by the process of dissolving within the cytoplasm or by extrusion. Variants of metarubricytes include multilobulated or clover-leaf nuclei, multiple fragments of varying size, single fragments called *Howell-Jolly* bodies and nuclei protruding from the surface of the cell. Occasionally there may be one normal appearing nucleus and nuclear fragments in addition, which is explainable on the basis of an abnormal division of the nucleus with one of the divided parts undergoing disintegration before the other (Plate 2.III).

Cabot's Rings. Occasionally one sees in the cytoplasm of red cells delicately looped figures or rings which take a purplish stain and have no internal structure. These may encircle the outer margin of the cell but may be smaller. More than one may be found in a given red cell. The exact nature of these structures is unknown. One opinion is that

they are the remains of former nuclei, another that they represent cytoplasmic defects or artifacts. Cabot's rings are infrequent but, when found in the peripheral blood, are indicative of abnormal regenerative activity of the bone marrow (Plate 2.III).

Stippled Erythrocytes. Red cells may contain multiple bluish dots or granules of varying size and number. Such cells are said to be stippled or to show basophilic aggregation. Basophilic stippling may be seen in nucleated red cells and in polychromatophilic cells. Punctate basophilia is thought to be due to cytoplasmic abnormalities of a degenerative nature taking place in the early red cells. Stippled cells are often but not always demonstrable in lead poisoning and in malaria and may be found in numerous other conditions (Plate 2.III).

Schuffner's Granules. Red cells containing *P. vivax* frequently have in their cytoplasm reddish granules called Schuffner's granules. Cells parasitized and showing these granules are, as a rule, larger than normal.

Variations in Color (anisochromia). The variations in color may involve varying degrees of blue stain, varying degrees of red stain, or of mixtures of red and blue. Nuclear structures and abnormal granules also may be considered as factors in causing color variation.

The term normochromic is used for cells with the normal amount of hemoglobin, hypochromic for cells having a very pale center (central achromia) with only a narrow rim of hemoglobin, and hyperchromic for those with an increased amount of hemoglobin. Irregularities in hemoglobin distribution are usually due to abnormalities in shape of the cell but may also be due to degenerative changes or abnormalities of cell formation (Plate 2.III).

Variations in Size (anisocytosis). Cells of normal size are called normocytes, cells smaller than normal, microcytes, and those larger than normal, macrocytes. As a rule, cells that are defective in hemoglobin are also small and cells that have a greater amount of hemoglobin per cell are large (Plate 2.III).

Variations in Shape (poikilocytosis). Oval or elliptical cells are the most common abnormality of shape in blood dyscrasias. These cells occur in some individuals as an inherited trait. Cells with blunt filaments or single or multiple points are often present in severe anemias and are indicative of defective cell formation. Sickled cells in their most diagnostic forms are elongated cells with sharp double points which have U, S, or L shapes, but long straight forms with pointed ends (oat shapes), oval and elliptical cells, and target cells are also present in this disease. Target cells are thin erythrocytes with abnormal elevations,

which, when appearing in the center of the disc, give to the cell the appearance of a dark center, surrounded by a light zone and an outer ring of hemoglobin. Spherical cells have a dark center and a diameter less than normal.

Semilunar Bodies. Red cells may contain vacuoles. Semilunar bodies, which are light bluish structures of crescent shape, are considered as smudges of degenerate red cells. Transitions between vacuolated red cells and semilunar bodies are demonstrable.

Granulocytic (Myelocytic) Series, Normal Sequence

Myeloblasts. The primitive cell of the myelocytic series has the morphological characteristics common to all "blasts". There are no granules. These cells make up less than 1 per cent of the nucleated cells of the bone marrow (Plates 2.I and 2.II).

Progranulocytes (promyelocytes, premyelocytes). Progranulocytes differ from myeloblasts in that the nuclear chromatin is coarser, the nucleoli are less well defined, the cytoplasm is less basophilic and, in relation to the nucleus, more abundant. Non-specific granules in varying numbers are present. As a rule, the granules are dark and predominantly basophilic, but in some cells the granules are reddish or purple or there are mixtures of red and blue granules, which are not as yet sufficiently developed to tell whether they are to be eosinophilic, basophilic or neutrophilic (Plates 2.I and 2.II).

Progranulocytes constitute 1 to 5 per cent of the cells of normal marrow and are not found in the normal peripheral blood.

Neutrophilic Myelocytes. At this level neutrophilic granules have begun to develop, first appearing among the bluish granules as a small reddish area. In later myelocytes the reddish granules are evenly distributed throughout the cell. The nucleus of the myelocyte is round, the chromatin more coarse than that in the progranulocyte and nucleoli are no longer visible. Myelocytes are not present in the normal peripheral blood, but appear in numerous conditions in which there is stimulation of the marrow as in myelocytic leukemia and infections. In the bone marrow myelocytes of the neutrophilic type constitute 2 to 8 per cent of the nucleated cells (Plates 2.I and 2.II).

Neutrophilic Metamyelocytes (juveniles). Neutrophilic metamyelocytes resemble myelocytes but have a slightly indented nucleus. The nuclear characteristics are intermediate between a myelocyte and band form (Plates 2.I and 2.II). Metamyelocytes of the neutrophilic type make up 5 to 15 per cent of the nucleated cells in the normal marrow and are occasionally seen in the circulating blood.



PLATE 2.III. Morphology of Red Blood Cells (Wright's Stain)

First Line, left to right—Rubriblast, prorubricyte, rubricyte, metarubricyte, diffusely basophilic erythrocyte, erythrocyte.

Second Line—Stippled metarubricyte, metarubricyte with fragmenting nucleus, metarubricyte extruding nucleus, metarubricyte with nuclear fragments, diffusely basophilic erythrocyte with Howell-Jolly body, stippled erythrocyte.

Third Line—Reticulocyte stained with brilliant cresyl blue and Wright's stain, Cabot's ring, platelet on erythrocyte, malaria ring, artifact, target cell.

Lower Line—Hyperchromic macrocytic erythrocytes, normochromic normocytic erythrocytes, hypochromic microcytic erythrocytes.

Neutrophilic Band Cells (N. non-segmented, N. non-filamented, N. stabs). This type of cell is slightly larger than the mature neutrophils. The nucleus is indented more than half of the width of the nucleus and often is in the shape of a bent stick. There may be dark pyknotic areas at the horns of the nucleus. The chromatin of the nucleus is condensed in plaques. The cytoplasm stains reddish and the granules are fine, evenly distributed and takes a neutral stain (Plates 2.I and 2.II).

Non-segmented neutrophils are the predominant cell in the bone marrow, ranging from 10 to 40 per cent. In the circulating blood these cells constitute 2 to 10 per cent of the leukocytes.

Neutrophilic Segmented (filamented neutrophils, neutrophilic polymorphonuclears, polymorphonuclear neutrophilic granulocytes, heterophiles). These cells differ from the neutrophilic band forms in that the nucleus is separated into lobes which are connected by tiny filaments or strands, rather than by wide bands. The nucleus is pyknotic. The cytoplasm stains pink and the granules are neutrophilic. The size is approximately twice that of the red cells (Plates 2.I and 2.II and Fig. 2.2).

Segmented neutrophils are often difficult to distinguish from non-segmented forms when the smear is thick or when the lobes overlie each other. If the margin of a given lobe can be traced from one side across the isthmus and is continuous with the margin on the other side, it is to be assumed that the lobes are superimposed and that the nucleus is segmented even if the filament is not visible. In case of doubt, the borderline cells are called segmented neutrophils.

Segmented neutrophils comprise 10 to 30 per cent of the marrow cells and 50 to 70 per cent of the leukocytes in normal blood.

Eosinophils (polymorphonuclear eosinophilic granulocytes, acidophils). Eosinophils develop from progranulocytes and pass through the myelocyte, metamyelocyte, band and segmented forms as do the neutrophils. The percentage of eosinophils is so small in the usual differential count that no useful purpose is served by separating the various developmental stages, hence it is recommended that all eosinophils be grouped under a single heading.

The eosinophils as seen in the peripheral blood are about the same size as neutrophils and usually have a band or two-lobed nucleus. The granules are spherical, uniform in size, usually evenly distributed and seldom overlie the nucleus. In good stains the granules are bright red and on focusing up and down a distinctive highlight can be demonstrated on each granule (Fig. 2.2). Sometimes when the stain is unsatisfactory

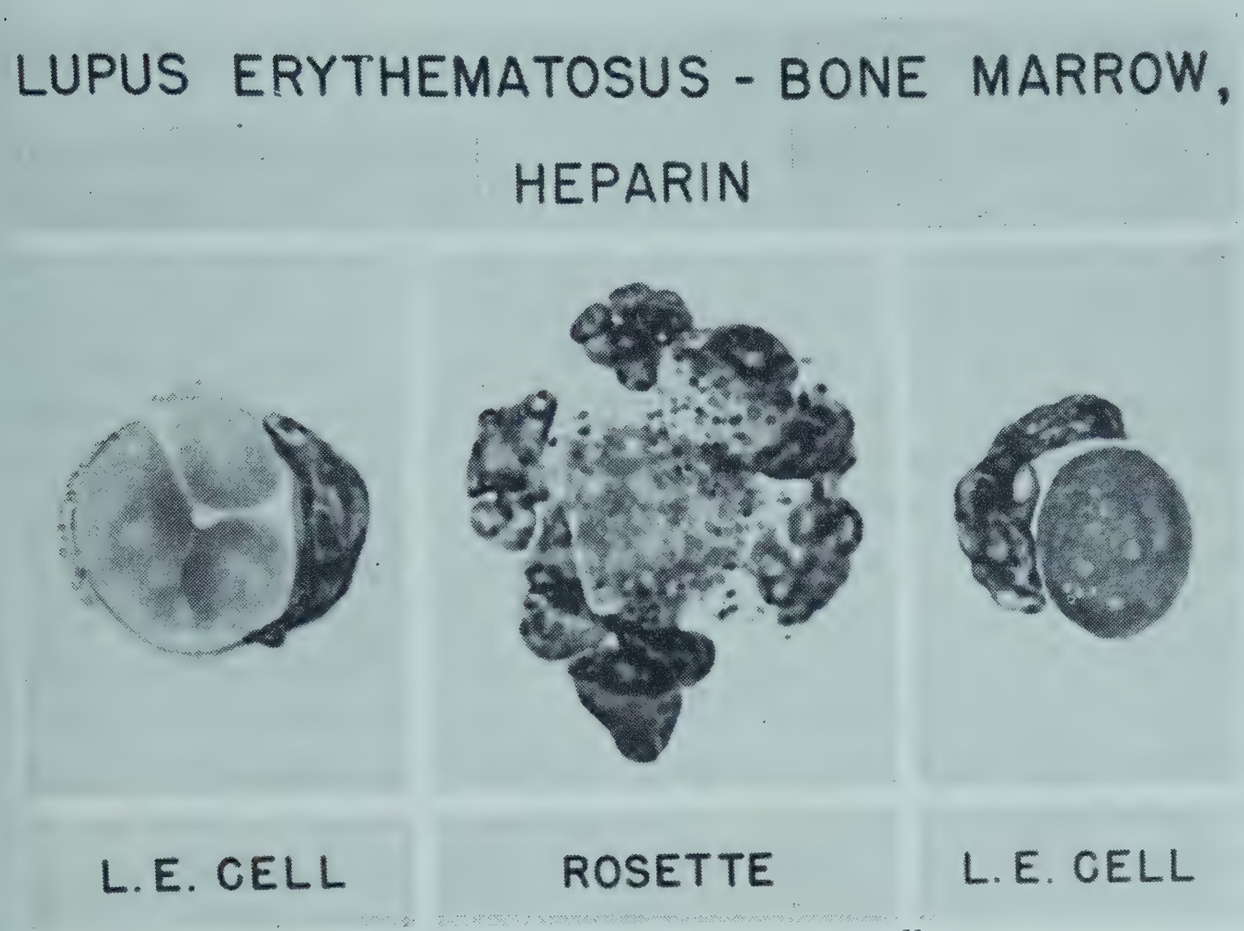


Fig. 2.3. Lupus erythematosus cells

Lymphocytic Series, Normal Sequence

Lymphoblasts. The morphology of the lymphoblast is similar to other primitive cells (Plate 2.II and Fig. 2.4).

Prolymphocytes (early lymphocytes). In the early lymphocytes the nucleoli are indistinct, the chromatin structure is moderately coarse, the nucleus round and the cytoplasm blue. These cells are found in

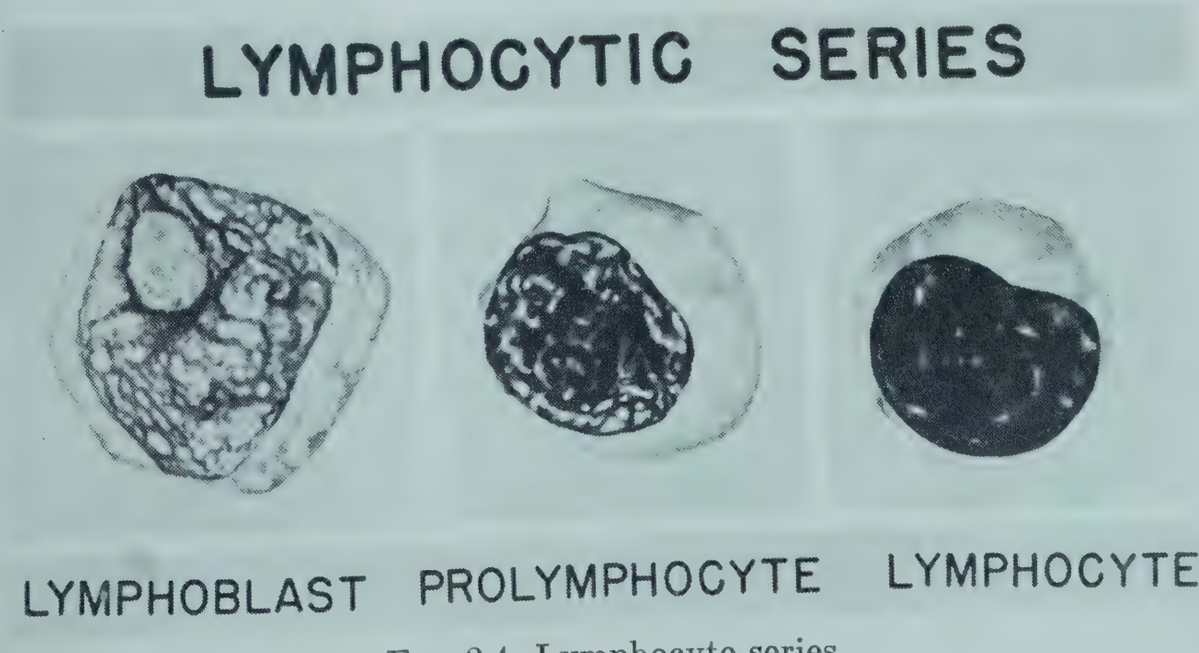


Fig. 2.4. Lymphocyte series

conditions in which there is an increase in lymphocytes in the peripheral blood (Plate 2.II and Fig. 2.4).

Lymphocytes. Mature lymphocytes constitute 20 to 35 per cent of the leukocytes in the circulating blood, or from 1,000 to 4,000 per cu. mm. In children the number is greater. Lymphocytes make up about 5 to 20 per cent of the nucleated cells in bone marrow smears.

The size of lymphocytes varies widely. The majority in normal blood are slightly larger than the red cells. These small lymphocytes have a round or slightly indented nucleus. The nucleus stains darkly and there are darker plaques or areas of condensed chromatin separated by lighter areas. There is a condensation of chromatin at the periphery of the nucleus and a perinuclear clear zone. There is a narrow rim of bluish cytoplasm. Intermediate and large lymphocytes are also present in normal blood. These cells have the same type of nuclear structure as the small lymphocytes. Maturity of the cell is evidenced by the fact that there is a relatively large amount of cytoplasm, which is a light robin's-egg blue. In about a third of the large lymphocytes a few unevenly distributed purplish-red granules, the so-called azurophilic granules, are found. No clinical purpose is gained by attempting to list the lymphocytes according to the large and small forms in the routine differential count, but in cases of lymphocytosis, it is well to make a note of the predominance of one form or the other. The lymphocytes are larger and the percentage of large forms greater in thin smears than in thick films, which is probably due to the fact that lymphocytes are easily flattened by slight pressure. Lymphocytes are also readily indented by neighboring red cells, causing them to have pointed or holly-leaf shapes.

Pathological Lymphocytes

Vacuoles are sometimes seen in the nucleus and cytoplasm of lymphocytes, indicating degenerative changes. Very large lymphocytes, lymphocytes with folded, dark blue or foamy cytoplasm and lymphocytes resembling monocytes and plasma cells are found in benign lymphadenitis (infectious mononucleosis).

In lymphocytic leukemia the lymphoblasts and early lymphocytes often have indented nuclei.

Monocytes

Monoblasts. Primitive cells of the monocytic series are not identifiable in normal individuals. In monocytic leukemia the stem cells have characteristics similar to other blasts (Plate 2.II).

Promonocytes (early monocytes). These cells have nuclear and cytoplasmic characteristics which are intermediate between the monoblast and mature monocyte. Linear chromatin pattern, indentations and folds in the nucleus, pseudopods and finely granular bluish cytoplasm are the rule (Plate 2.II).

Monocytes (large mononuclears, endothelial leukocytes). The monocytes vary in size from 15 to 20 microns. As a rule, they are larger than the neutrophils in the same microscopic fields. The cytoplasm is large in relation to the nucleus. The cell often is oval in shape or has large blunt pseudopods or multiple small rounded cytoplasmic protrusions, which, as a rule, are devoid of granules. The nucleus is usually eccentric and indented, but may be round, oval or lobulated. Folds or depressions of the nucleus, resembling the convolutions of the brain, are often present. The nuclear chromatin is coarse and has a linear pattern, similar to that seen in the histiocyte. Peripheral condensation of nuclear chromatin and a perinuclear clear zone are not as striking as in the large lymphocyte. The cytoplasm is finely granular, giving it a ground glass or opaque appearance. The granules vary in prominence and are usually neutrophilic, but there may be in this cell, as in all other cells, bluish granules.



FIG. 2.5. Variations in the morphology of monocytes

Digestive vacuoles are demonstrable in this cell and may contain phagocytized red cells, nuclear fragments, pigment, bacteria or other particulate matter. The cytoplasm has a bluish gray color which is quite distinctive and of value in differentiating monocytes from neutrophils (Plate 2.II and Figs 2.5 and 2.6).

Monocytes comprise 1 to 6 per cent of the leukocytes of the circulating blood but are rare in the bone marrow.

The morphological differences between atypical monocytes and early or atypical cells of the lymphocytic or early myelocytic series are slight, and particularly is this true in stem cell leukemia and diseases in which there is hyperplasia of the hematopoietic tissues. Folds and indentations of the nucleus, linear chromatin patterns, vacuoles in the cytoplasm, blunt cytoplasmic protrusions, evidences of phagocytosis, transitions between this cell and typical monocytes and histiocytes constitute the principal criteria used in placing atypical cells in the category of monocytes. Aggregation of the nuclear chromatin in plaques, condensation of chromatin at the periphery of the nucleus, perinuclear clear zone, lack of evenly distributed granules, pointed cytoplasmic projections and similarity of the cell to typical lymphocytes aid in the identification of lymphocytes. Myelocytes and metamyelocytes have well-defined granules, the nucleus is relatively large in relation to the cytoplasm, folds in the nucleus are absent, the cell is usually round with smooth edges, and transitions occur between progranulocytes and lobulated neutrophils (Figs. 2.5 and 2.6).

Plasmocytes

Plasmoblasts. Undifferentiated cells of the plasmocytic series (Fig. 2.7) are demonstrable in plasma cell myeloma (plasmocytoma, multiple myeloma) but are not recognizable in normal material. The characteristics of plasmoblasts are the same as other "blasts." As in any form of malignancy, the nucleoli of plasmoblasts are often very large. This feature has led to the use of the term "myeloma cell" for the early cells with conspicuous nucleoli seen in multiple myeloma, but the use of such a term is unwarranted.

Proplasmocytes. The proplasmocytes have nuclear chromatin structures intermediate between the "blast" and mature forms (Fig. 2.7).

Plasmocytes (plasma cells). Plasmocytes are characterized by an oval or elongate shape, a round, relatively small nucleus which is pyknotic and eccentrically placed, a dark purplish-blue cytoplasm and a conspicuous relatively unstained area next to the nucleus (Fig. 2.7). Un-

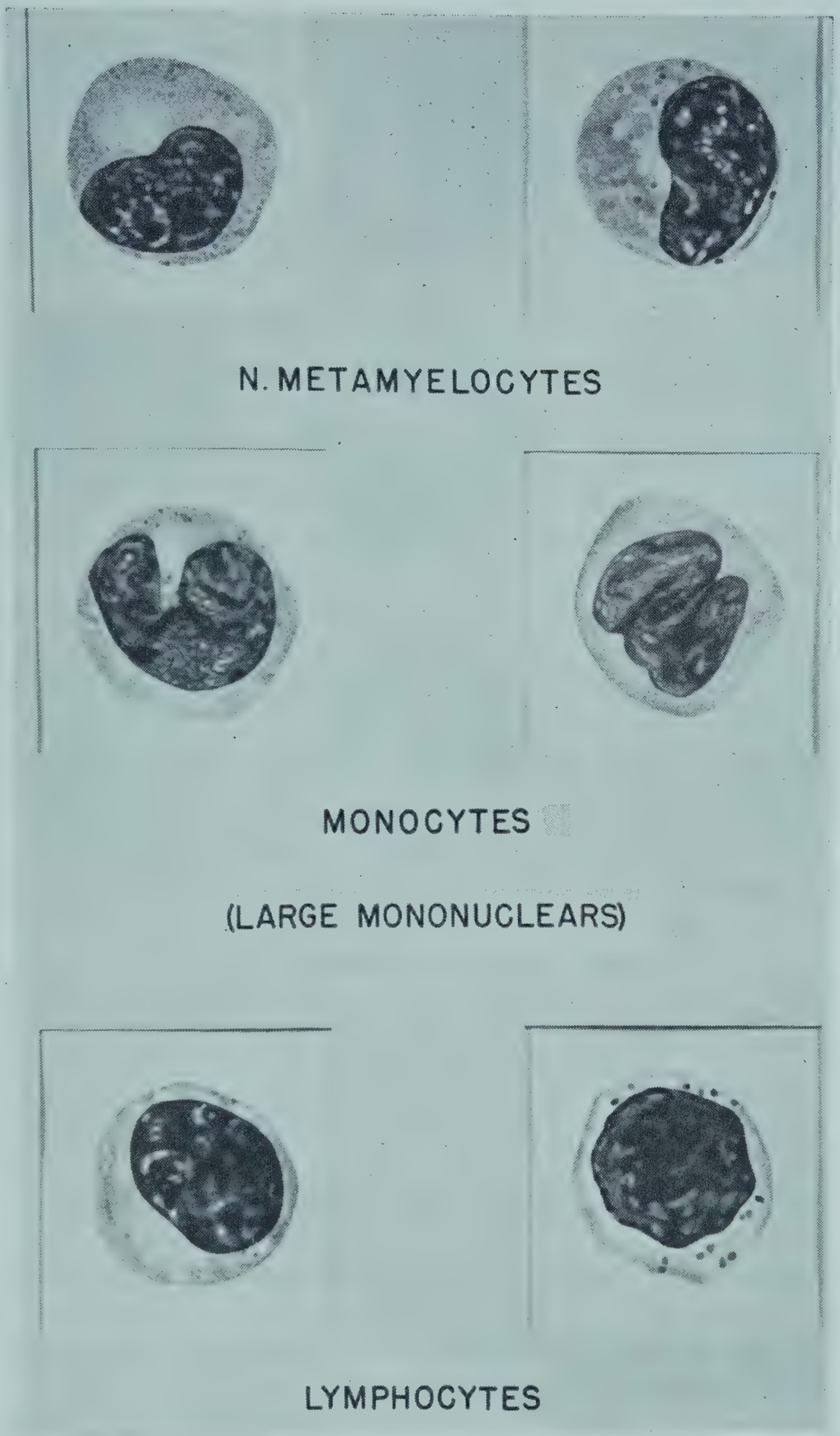


FIG. 2.6. Neutrophilic metamyelocytes, monocytes and large lymphocytes

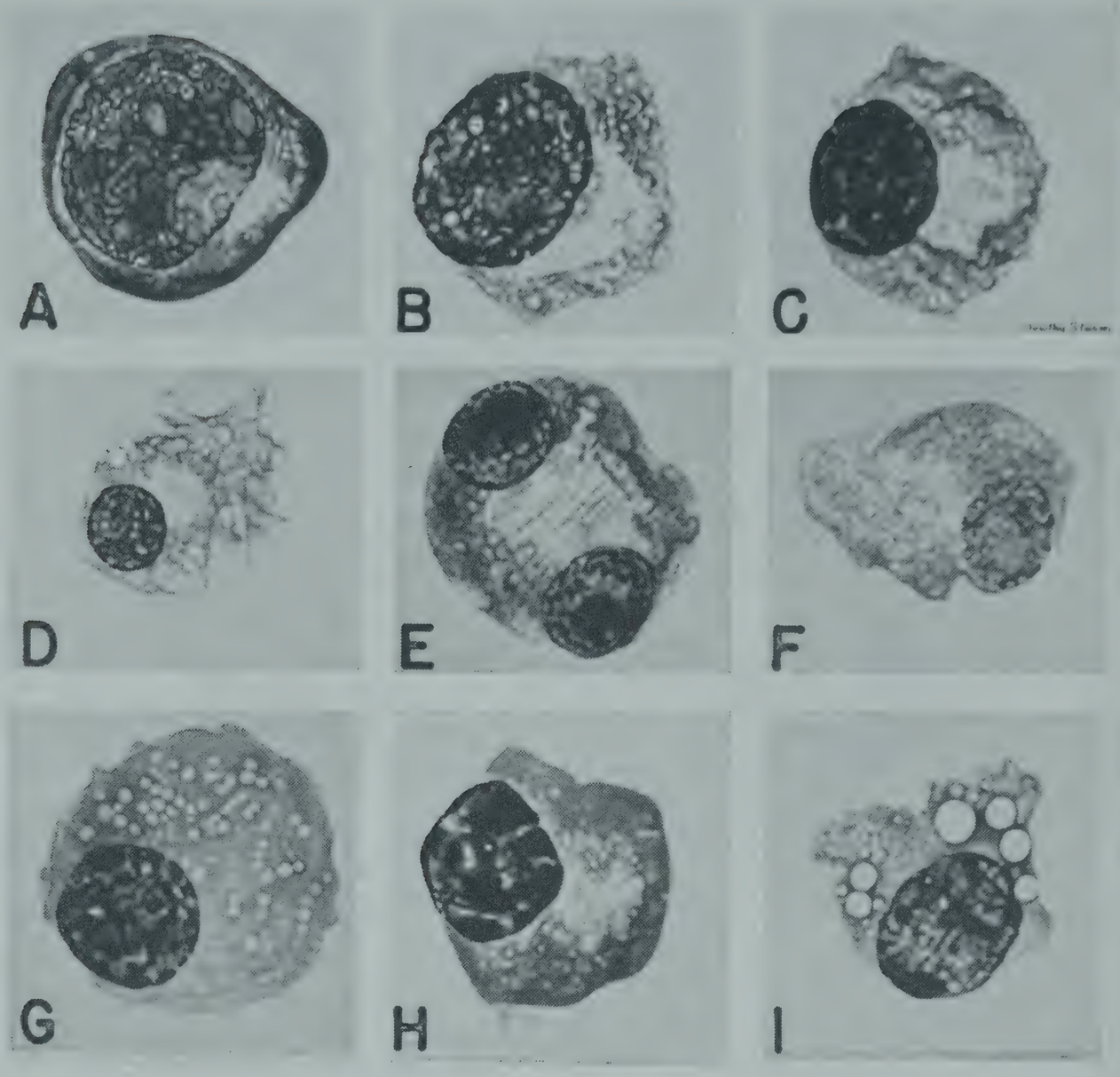


FIG. 2.7. Plasmocyte variants

A. Plasmoblast.
B. Proplasmocyte.
C. Plasmocyte.
D. Plasmocyte showing fibrillar structure.
E. Proplasmocyte with double nuclei.
F. Proplasmocyte with cytoplasmic streamers and ragged margins.
G. Plasmocyte with Russell bodies.
H. Plasmocyte.
I. Plasmocyte with unstained globular bodies.

stained globules within the cytoplasm give to the cell a foamy appearance. In some cells there are reddish-staining homogeneous masses which may be in the form of well defined globules, called "Russell bodies" or "eosinophilic globules," or in the form of pools of reddish material which seems to be exuding from the cell margin or irregularly dispersed within the cytoplasm. The peculiar brilliant and translucent quality of the plasmocyte cytoplasm is probably due to the presence within the blue cytoplasm of these unstained and reddish structures.

In thin preparations one can see a fibrillar structure within the cytoplasm or delicate lines which extend outward from the cell in a nebulous manner. The shape of the plasma cells in bone marrow preparations is characteristically irregular with long cytoplasmic streamers, but this feature is less striking in the peripheral blood cells.

The so-called "cart wheel nucleus" which has been described as a characteristic of the plasma cell in tissue sections is not seen in Wright's stained smears.

Cells with double nuclei are fairly frequent.

Plasmocytes do not usually have granules, but there are rare cases of plasma cell myeloma in which cellular structures having well defined purplish spherical and rod-like structures are seen. In thicker preparations one may easily mistake the uneven stain caused by the globules within the sponge-like cytoplasm for granules, or see true myelocytes or progranulocytes mixed with plasma cells and mistakenly interpret these granulocytes as granular plasmocytes.

In the bone marrow plasmocytes are often found in small groups clustered around an endothelial or undifferentiated stem cell. The finding of such a nest with an associated endothelial cell is evidence of normality and against the diagnosis of malignancy.

Plasmocytes closely resemble immature nucleated red cells for both have a reddish-blue cytoplasm, a round nucleus and similar chromatin structure. Plasmocytes as a class have more cytoplasm in relation to the size of the nucleus. The eccentric nucleus, the unstained area near the nucleus, the foamy and fibrillar cytoplasm and ragged margins aid in identifying the cells as plasma cells.

In many preparations there are individual cells which have dark cytoplasm but otherwise resemble early lymphocytes. In case of doubt these cells should be classified as lymphocytes or placed in an atypical column.

Megakaryocytes and Thrombocytes

Megakaryoblasts. Megakaryoblasts resemble the undifferentiated stem cell in that they have a round, relatively large red-staining nucleus, fine chromatin pattern and nucleoli. The cytoplasm is hyaline and stains light blue. The outer layers tend to differentiate as darker blue, often vacuolated zones. Cytoplasmic masses appear to be breaking off at the margin of the cell. Non-granular megakaryocytes in mitosis are classed as megakaryoblasts. Less than 1 per cent of the megakaryocytes are megakaryoblasts (Plate 2.II).

Promegakaryocytes (early megakaryocytes). The nuclei of these cells have an early type of chromatin structure, but are indented or multilobulated. The cytoplasm stains blue and contains a variable number of fine bluish granules. Cytoplasmic buds are usually demonstrable. This cell type constitutes 5 to 10 per cent of the megakaryocytes as seen in normal marrow, but may be relatively increased in thrombopenic states (Plate 2.II).

Megakaryocytes and Metamegakaryocytes (mature megakaryocytes). These cells are often more than 100 microns in diameter. The cytoplasm is plentiful and is characterized by relatively coarse granules in the megakaryocyte. Part of the granules in the cytoplasm are aggregated in small ill defined clumps or as well defined masses (thrombocytes) in the metamegakaryocyte. The thrombocytes usually are most numerous at the periphery of the cell. The nucleus of mature megakaryocytes is indented or lobulated, has an intermediate or mature chromatin pattern and does not contain nucleoli. The nucleus may appear as a naked lobulated structure or may have small numbers of mature thrombocytes attached to it. Fragments of megakaryocytes with attached thrombocytes are occasionally seen in peripheral blood in myeloid leukemia and other hyperplastic states, but larger forms are not seen because the cells are too large to circulate (Plate 2.II).

The number of megakaryocytes in normal bone marrow is approximately 2 to 5 per 1000 nucleated cells. In tissue sections the megakaryocytes usually appear as isolated cells and seldom as groups, but in marrow smears they often are grouped in thicker portions of the film and in fibrin aggregates.

In thrombocytopenic conditions the megakaryocytes may be present in normal or in increased numbers, but are not producing granular thrombocytes in the normal manner. In such conditions a megakaryocyte differential count with a subdivision of the megakaryocytes into separate columns "without" and "with" thrombocyte production is recommended.

Thrombocytes in the peripheral blood exist in varying numbers, depending on the method used for counting (see Chapter 4). They appear in smears of bone marrow and of peripheral blood stained by polychrome methylene blue-eosin stains as bluish hyaline cytoplasmic masses with variable numbers of small bluish granules, usually grouped toward the center (Plate 2.II). Their size varies from 2 to 4 microns, but in pathological conditions, smaller forms of thrombocytes or forms larger than white cells may be demonstrated. The shape of the cell on careful focus-

ing is often seen to be irregular with cytoplasmic filament projecting from the margins. Thrombocytes tend to stick to each other, often forming large aggregates of various shapes and constituted of hundreds of individual units. Pathological thrombocytes may have vacuoles or defective granular structure.

Other Cells Found in the Bone Marrow

In addition to the above described cells of the peripheral blood and their progenitors in the hematopoietic tissues there are other cells which are observed in small numbers in normal bone marrow and are increased in certain pathological states. These cells, like all other marrow cells are derived from the undifferentiated mesenchymal cells or hemohistioblasts. As a class these cells are fixed tissue cells which are not readily aspirated and which can be better seen in microscopic sections.

Reticular cells (reticulum cells). The anatomist and pathologist visualize the reticular cells as supporting or connective tissue elements which have multipointed cytoplasmic projections and are connected and associated with net-works of interlacing fibers which are best visualized by impregnation with silver salts (argyrophil fibers). To the hematologist the reticular cell is more of a concept than a recognizable morphological entity, for the cell is a fixed tissue cell which is rarely seen in smears and has no identifying features when stained with Wright's or comparable blood stains. It is recommended that the term "reticular" and "reticulum" be reserved for cells which can be shown by special staining methods to have a specific type of fiber, and that the word not be used as a synonym for "stem cell," "hemohistioblast" or "undifferentiated mesenchymal cell."

One occasionally encounters in marrow material large cells with primitive types of nuclei which have long cytoplasmic streamers and a few ill-defined granules and fibrillar cytoplasmic structures. It is probable that such cells represent transitions between the undifferentiated mesenchymal cells (stem cells) and granular or phagocytic histiocytes, and may be designated as prohistiocytes (Plate 2.IV).

Granular histiocytes (histiocytes, reticular cells, reticulo-endothelial cells, Ferrata cells). This cell is one about which there has been much controversy. Few facts are established concerning its function or its relationship to other fixed tissue cells and to wandering macrophages, monocytes and progranulocytes. In bone marrow material stained with Wright's the cell has distinctive morphological features.

The granular histiocyte is characterized by prominent and well de-

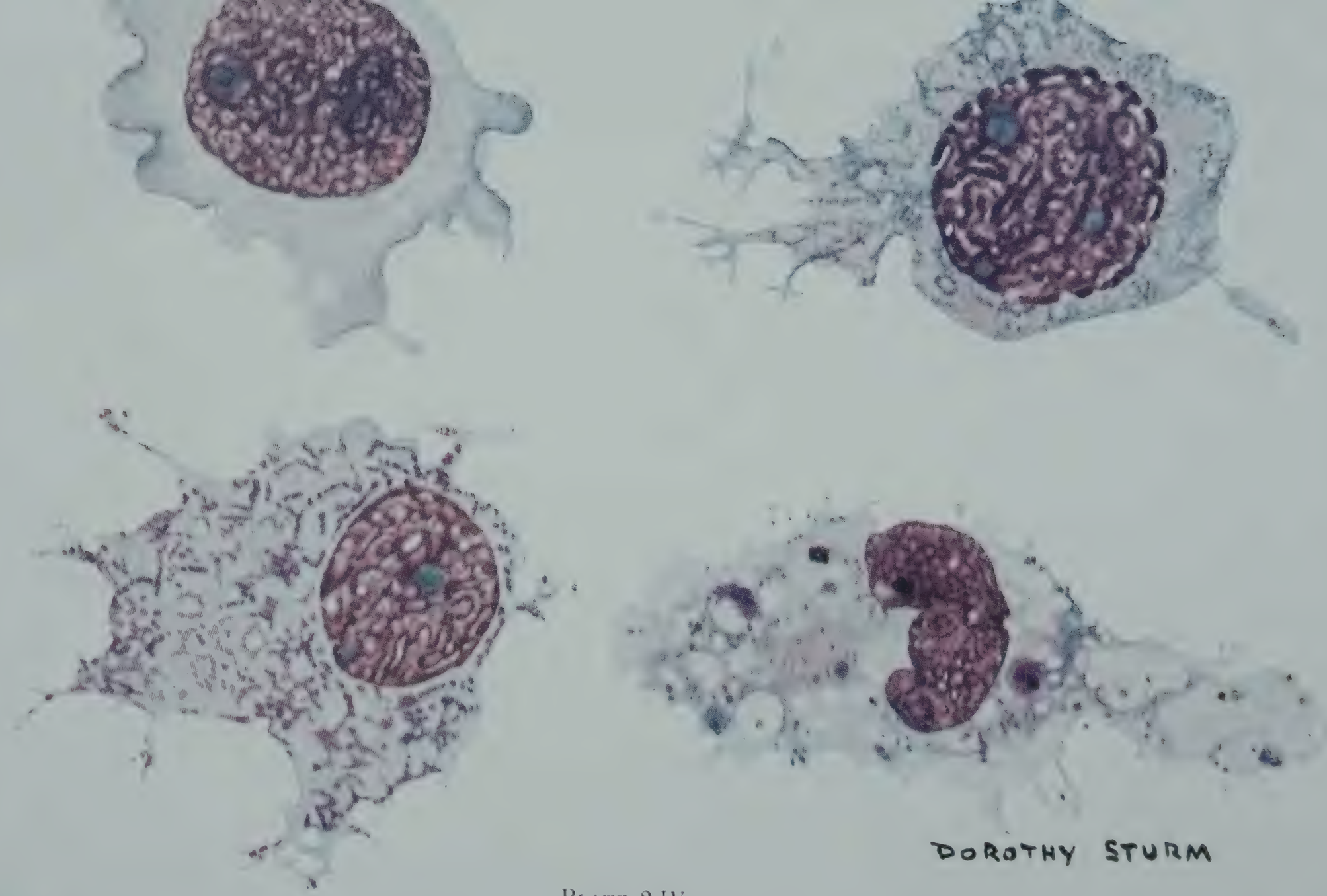


PLATE 2.IV

Top Left—Undifferentiated mesenchymal cell or stem cell.
Top Right—Fixed tissue cell with minimal granulations and fibrillar structure (? Pro-histiocyte).
Lower Left—Granular histiocyte. Note linear type of red granules.

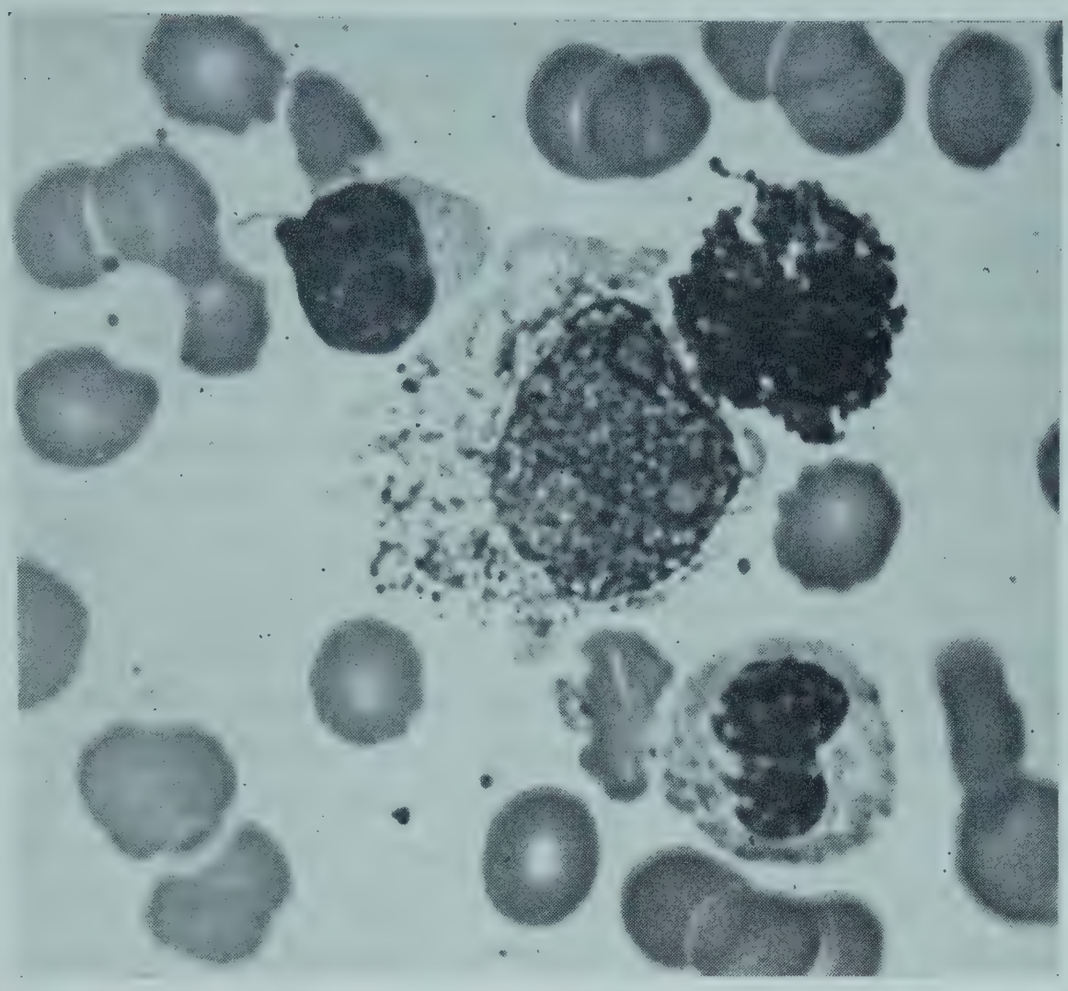


FIG. 2.8. Granular histiocyte. Note large size, irregular shape, linear granules, coarse chromatin structure of nucleus and nucleoli. Other cells are lymphocyte, basophil and neutrophil. From sternal marrow, × 1500.

fined granules which vary in color but are usually bright red. These granules vary in size, shape and number. Some of the granules are peculiar in that they are linear and bacilliform or appear as beaded chains (Plate 2.IV and Fig. 2.8). In most of the cells the granules have no distinctive pattern, but in the more elongate cells the filamentous granules tend to be parallel to each other and to the margins of the cell. Granular histiocytes vary in size, but as a class are two to four times larger than neutrophils. There is ample cytoplasm and a large nucleus which is round and has well defined coarse chromatin structure and prominent nucleoli. The shape is characteristically irregular with nebulous cytoplasmic streamers. We have yet to see a granular histiocyte with well defined red-staining linear granules which contained any recognizable phagocytized material.

Many consider that the above described cell is an artefact or a degenerated cell. It is true that the cell is misshapen and that the nuclear membrane is often partially torn with the characteristic coarsening of chromatin which occurs when the nuclear membrane is injured, but it is easy to find intact cells in which there is no apparent injury. The

cellular structures and particularly the red staining granules are so sharp and distinctive that we cannot accept the concept of this cell being degenerate, although we do not know exactly where it belongs in the blood cell or connective tissue family.

Phagocytic Histiocytes (fixed and wandering macrophages, clasmatocytes, phagocytic reticular cells). These cells as a class are large and have a large amount of cytoplasm and a nucleus which is relatively small and resembles that seen in monocytes. The cells are vacuolated and often contain phagocytized material such as debris, nuclear fragments, whole cells, or blood pigment (Plate 2.IV). The cell may exist as a fixed tissue cell or it may have slow ameboid activity. Seldom is it seen in smears of peripheral blood for the cell is too big to get through the capillaries readily. Mesenchymal cells with phagocytic properties are the cells which are thought to be involved in histiocytosis associated with the lipoid storage diseases and are the cells which harbor histoplasma capsulatum, leishmania donovani, and blood and other pigments.

The separation of histiocytes into phagocytic and non-phagocytic forms as given above for descriptive purposes is open to criticism, for a given cell may be non-phagocytic at one time and later may acquire phagocytic properties or vice versa.

Endothelial Cells. In bone marrow material one occasionally sees small capillaries with endothelial cells arranged in characteristic organoid patterns. These cells have oval nuclei and elongate non-granular cytoplasm. Individual endothelial cells are not identifiable.

Tissue Basophils (mast cells, heparinocytes). These cells are fixed tissue cells which are adjacent to blood vessels and are thought to be the source of heparin. They are characterized by very deeply staining, bluish-black granules which are round, uniform in size, numerous, and tend to obscure the nucleus.

Fat Cells (lipocytes). These cells in their earliest stages resemble plasmocytes in that they have a blue cytoplasm, an eccentric nucleus and globules of unstained material in the cytoplasm giving it a foamy appearance. As the cell matures the fat globules become larger and coalesce. As usually seen, the fat cells are grouped fixed tissue cells which are characterized by a large pool of non-stained material through which a fibrillar structure can be seen, and a nucleus which is shoved to one side and flattened, giving a signet ring appearance. In tissue sections the nucleus is not always discernible. In making smears many of the fat cells are ruptured and appear as free fat. The cells from which the fat comes are not identifiable.

BONE MARROW EXAMINATION

Indications

The examination of the bone marrow is indicated in any disease characterized by abnormalities of the blood in which the diagnosis cannot be established by the usual clinical examination and laboratory tests. The examination is particularly indicated in those conditions in which there is a decrease in cellular elements, as in leukopenia, thrombocytopenia and non-regenerative anemia. Other general indications are unexplained lymphadenopathy, splenomegaly or hepatomegaly; unexplained fever, cachexia or weight loss; leukemoid reactions and osteolytic lesions.

Specific diseases in which characteristic cytological changes are demonstrable in a high percentage of cases are plasma cell myeloma, hemosiderosis, lupus erythematosus and the histiocytic lipoid storage diseases. The etiologic agent may be demonstrable in such diseases as histoplasmosis, leishmaniasis and malaria.

In thrombocytopenic states the marrow examination is useful in differentiating leukemia and hypoplastic states from idiopathic thrombocytopenic purpura. Splenectomy should not be performed unless there are ample numbers of megakaryocytes. In neutropenia and pancytopenia with splenomegaly, splenectomy is not advisable unless the marrow is cellular.

From the standpoint of the patient, the marrow study is most valuable when no abnormality is revealed and serious diseases such as leukemia, myeloma or aplastic anemia are excluded.

The diagnosis of metastatic malignancy can sometimes be established from spreads of aspirated marrow, but metastatic tumors are unevenly distributed and negative findings have no value. The finding of malignant cells in the marrow cavity is helpful in proving that a given tumor is inoperable.

When the marrow aspiration is combined with a section of the clot or when a marrow biopsy is taken, the diagnosis of osteosclerosis, malignancy, Hodgkin's disease, or infectious granuloma such as tuberculosis, undulant fever, sarcoidosis or infectious mononucleosis can sometimes be made. In any febrile illness or suspected granuloma some of the aspirate should be inoculated into culture media, for occasionally it is possible to demonstrate etiologic agents even when the blood cultures are negative.

In diseases such as leukemia with high white cell counts, pernicious

anemia, hereditary spherocytosis, sickle cell anemia and anemia due to iron deficiency, the bone marrow changes are characteristic, but the diagnosis of these diseases can, in most instances, be made without resorting to bone marrow puncture. In anemia or leukocytosis of undetermined or doubtful type, the marrow examination often aids in the differential diagnosis and gives a clue to the form of therapy indicated.

Bone marrow examination is of little value in the diagnosis or differential diagnosis of chronic myelocytic leukemia in the early stages of the disease or in conditions characterized by eosinophilia or in the erythrocytoses.

In consultation practice the patients referred usually have obscure blood dyscrasias and plans should be made to perform marrow examination on such patients as a part of the initial survey; but even in such patients, the procedure should be postponed until the blood counts have been completed and a preliminary examination of the blood smear made, for often leukemia or other diseases can be readily diagnosed by peripheral blood studies. In case of doubt concerning indications, and in particular when the patient is in the hospital or lives in another town, it is better to proceed immediately with the marrow puncture so that the final diagnosis can be made as soon and as accurately as possible, specific treatment started, and time and money saved.

Marrow examinations are often performed and justified in teaching and research centers on patients whose diagnosis can be made by routine examinations, but in general the procedure should not be done if the diagnosis can be made with certainty by simpler means. It is to be remembered that marrow examination like any other specialized laboratory procedure is expensive, causes pain, and is not without danger. The operation should not be attempted unless the operator is trained, unless facilities are available for processing the material and a pathologist or other trained person is available to interpret the findings and to give the patient the maximal benefit.

The only contraindication to bone marrow biopsy or aspiration is hemophilia.

Sites

The bones preferred for marrow puncture are the sternum, the ilium, the spinous processes of the vertebrae and the upper end of the tibia. Marrow obtained from any of these sites has essentially the same cellular composition.

Tiba. For newborn infants and children under the age of four, the

tibia is the site of choice. The needle is inserted at right angles to the tibia, about 3 cm. below the knee cap in the midline. Local anesthesia is not recommended for this age group for the injection of procaine prolongs the procedure, and makes two skin punctures necessary instead of one. Young children seem to resent restraint more than the temporary pain of puncture, hence the sooner the procedure is completed the better.

Ilium. For apprehensive patients, for patients on whom multiple punctures have to be performed, in suspected bone metastases and on children above the age of four who are difficult to manage, the puncture of the iliac crest is the site of choice. There is more likelihood of obtaining marrow diluted with peripheral blood when the ilium is used than when the sternum is used. The patient lies on his back or side. The needle is inserted at right angles to the plane of the ilium, several centimeters below the superior crest and about 4 cm. posterior to the anterior superior spine. "The posterior iliac crest is preferred by some for it is psychologically less traumatic and affords representative samples of bone marrow" (California Med. 77: 138, 1952).

Sternum. For adults the sternum is the site of choice because the bone is near the surface, the cortical bone is usually thin and the marrow cavity contains a maximal number of cells and a minimal amount of fat and trabecular bone. The patient lies on an examining table or firm bed with a small pillow placed under the shoulders to aid in raising the chest and lowering the head. The chest is shaved if necessary. The puncture may be made near the midline of the body of the sternum at the level of the third interspace, or slightly above or below this point. The space directly opposite the rib insertions should be avoided for these areas may be cartilaginous. The manubrium may be used. The width of the sternal marrow cavity in adults is approximately 1 cm. and the thickness of the cortical bone about 1–2 mm., but there is considerable individual variation. By entering the bone at a 45 degree angle, the margin of safety is increased. Long needles without guards are to be avoided, for accidents due to puncture of the heart or major vessels have occurred. As a rule the guard is set 3 to 4 mm. above the skin when the point of the needle is touching the periosteum.

Spinous Process. The spinous processes of lumbar vertebrae furnish another satisfactory area for aspiration and are preferred by some because the patient does not see the needle and vital structures are not endangered. The use of the spinous processes is advocated when multiple punctures are to be performed. The patient lies on the side with

the back arched or face down with a pillow under the abdomen. The third or fourth lumbar vertebra is usually chosen, but other vertebrae may be used. The patient may sit up for this procedure.

In certain patients there may be localized pathology as revealed by tumefaction or roentgenographs in ribs, clavicle, ischium or other bones. Aspiration at such sites may be made.

At autopsy, blocks of tissue should be removed from the vertebral body or sternum and in special cases also from the ribs and long bones. Because cytolysis occurs rapidly after death and degenerative changes occur faster in the neutrophilic leukocytes than in the other cell types, the study of smears at autopsy is of limited value.

Supplies and Equipment

Needles. Needles used for marrow puncture should have tightly fitting obturators so that bits of tissue, spicules of bone and blood clots do not occlude the lumen. The needle should be sharpened with the stylet in place after each use. A short bevel is preferred. For sternal puncture 18 or 19 gauge needles are preferred to larger needles. Smaller needles are easier to push through the skin and cortical bone than are large needles and yield comparable and satisfactory marrow specimens.

The needle of choice for sternal puncture in adults is the 18 gauge Rosenthal needle, 1 inch long (B-D 460LNR) (Fig. 2.9). A needle stop or guard (B-D 418S) for controlling the depth of puncture may be used in conjunction with this needle. For stout individuals the guard should be removed before puncture because the needle with guard attached may not be long enough to get into the marrow cavity. For sternal puncture in children a set of smaller needles, 0.5, 1.0, and 1.5 cm. in length are useful. These may be prepared by filing off and sharpening 19 gauge spinal needles of the Barker type (B-D 461LNR).

For tibial, iliac crest and spinous process punctures longer and slightly larger and more sturdy needles are recommended. The Osgood (B-D 458LNR) and Rosenthal (B-D 460LNR), 16 gauge and $1\frac{5}{16}$ inches long, or the 16 gauge University of Illinois needle (V. Mueller and Co., Chicago) are the needles of choice.

The University of Illinois needle, developed by Limarzi and Bedinger, is a modification of the Klina-Rosseger needle which has long been a favorite with many hematologists, particularly on the continent. Desirable features of this needle are the large stylet head, making it easy to hold, an adjustable guard which controls the depth of puncture and creates a shoulder on which pressure can be exerted, a locking device

which prevents the stylet from being dislodged during the insertion of the needle, and a hub which extends above the surface of the needle and which fits standard glass or metal-tipped syringes (Fig. 2.9). When this needle is used, it is recommended that the point be inserted until it touches the periosteum. The guard is screwed down until it touches the skin, after which a number of turns are taken in the guard corresponding approximately to the number of millimeters depth of puncture desired in the bone. The needle is then pushed forward until the guard again engages the skin surface.

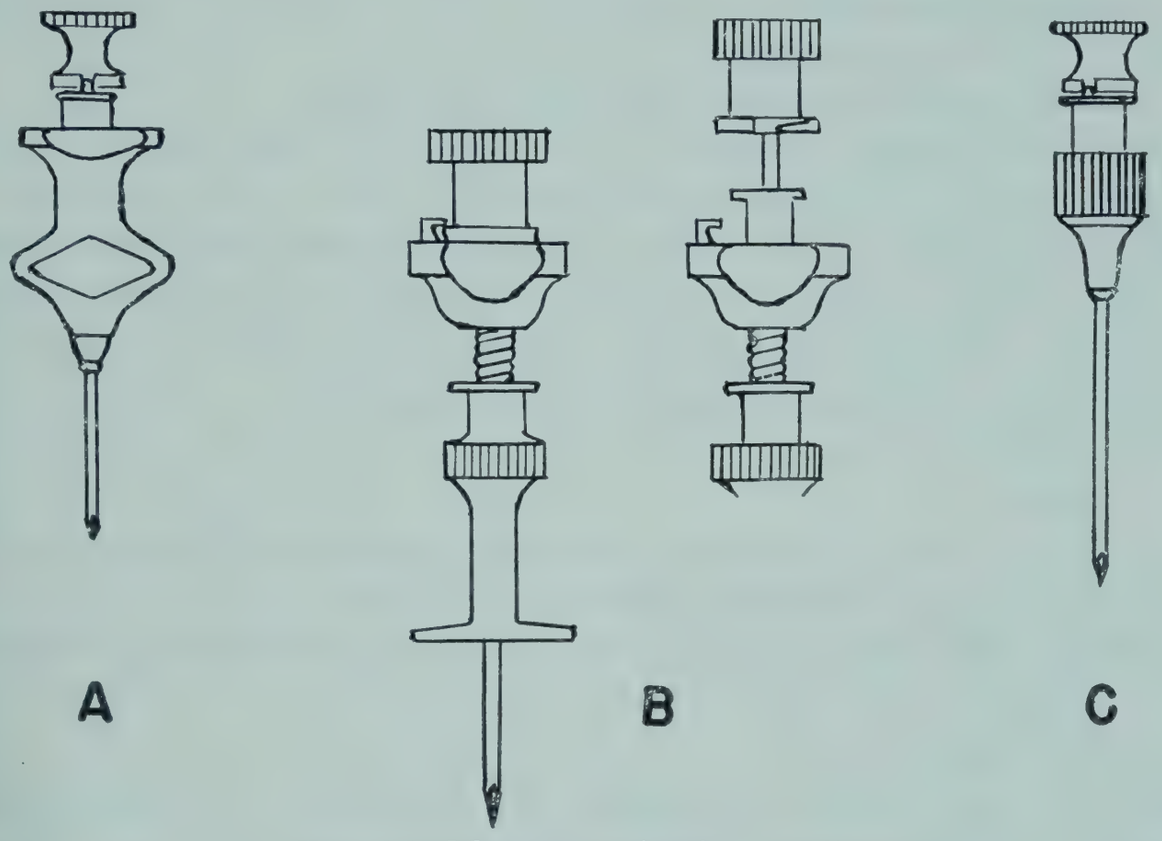


FIG. 2.9. Marrow aspiration needles
A. Rosenthal. B. University of Illinois (Limarzi). C. Osgood.

Syringes. For the aspiration of bone marrow a tight-fitting syringe of good quality is essential. The person preparing the tray and the doctor who uses the syringe should check the numbers on the barrel and adapter to see that they correspond. Twenty-milliliter syringes are preferred to small syringes for aspiration because a more forceful pull can be exerted by these syringes, and slight leakage of air around the needle hub will not cause failure. Syringes with metal tips are preferred to all glass syringes because it is easy to break off the glass tip in the hub of the puncture needle. It is well to have an extra sterile syringe wrapped in a separate paper in addition to the one on the tray. The syringe that is used for procaine should not be used to aspirate marrow because such solutions distort cytology.

Tray. Because fairly large needles are used and local anesthesia is indicated and because there is possibility of bone infection, it has been the custom to conduct the procedure as a minor surgical operation, using rubber gloves and isolating the operative field with sterile towels or drapes.

The list of equipment recommended for the bone marrow tray includes:

2 towels
1 emesis basin
8 cotton sponges
1 5 ml. glass syringe
2 24 gauge hypodermic needles, 1 inch long
2 bone marrow needles
1 20 ml. syringe with metal tip

The covered bone marrow tray is autoclaved and kept in readiness. Equipment not enclosed in the tray but accompanying the tray are:

1 pr. of sterile rubber gloves in paper wrappers
Procaine in rubber capped vial or in ampule
Alcohol
Iodine
Clean, new glass slides in slide box (Coverslips if desired)
Small bottle containing 10% formaldehyde
Hagedorn needle for puncturing finger and obtaining peripheral blood smear
Band-aids

The technique of marrow aspiration can be made as simple as venipuncture, the equipment needed being bone marrow needle, a syringe, skin disinfectant and cotton for cleaning the skin and smears for making the spreads.

Preparation of Patient

The fear and worry over the performance of the bone marrow aspiration or biopsy is often worse than the actual procedure; hence it is a good rule to prepare the adult patient with a sedative, such as pentobarbital sodium (0.1 to 0.2 gm.) or seconal (0.2 gm.) orally about an hour before the procedure. Barbiturates should not be used if the patient comes to the office unattended and has to drive a car afterwards. In particularly apprehensive patients who have a low threshold for pain, codeine (0.03 gm.) or morphine (0.01 gm.) may be given alone or in combination with barbiturates. For children a rectal suppository of

pentobarbital sodium (0.06 gm.) or a teaspoonful of phenobarbital elixir (15 mg.) or equivalent sedative helps in the management of the child and makes the ordeal less trying on the physician and parents. Morphine and other narcotics should not be given to children.

In patients admitted to the hospital for diagnostic study, the bone marrow examination should precede transfusions or specific medications, for such medications may mask the essential pathology. If a patient is suspected of having pernicious anemia and has been taking liver extract or equivalent or has been transfused previous to examination, the marrow examination should be deferred for 3 or more weeks.

Marrow Aspiration

Direct Smear Technique. Marrow punctures are preferably done in an examining room where facilities are convenient and an examining table is available. A good light is essential. A small table or surgical cart is needed so that the marrow puncture tray can be placed in a position convenient to the operator. In dealing with children it is preferable to have the assistant stand on the side of the examining table opposite the operator so that the child can be restrained without interfering with the operation.

The bone marrow tray is opened by the operator or his assistant. The operator washes his hands, dries them and puts on sterile gloves. He takes up one of the cotton sponges with a hemostat, and an assistant pours iodine over this sponge. The site of needle puncture is cleaned with iodine, followed by alcohol. Several towels are placed to protect the operative field. Approximately 2 to 5 ml. procaine is taken up in the small syringe and injected intradermally, subcutaneously and at the surface of the bone. A sponge is pressed over the injection site in order to cause the dispersion of fluid. The needle is now taken up and inspected for patency and to test the fit of the stylet. The needle is inserted through the skin down to the periosteum. If the guard is on the needle, this is now set. The needle is advanced with a boring motion. In sternal puncture the pressure should be guarded so that the needle on passing through the outer table of the sternum does not go through both tables into the mediastinum. As a rule the needle can be felt to "give" as it goes through the cortex, but this sensation may be absent. A needle which has entered the marrow cavity to a sufficient depth will be supported by the bony structure and will remain upright when the hand is removed. If the needle falls over, it is not in bone and must be pushed further. After the needle is inserted, the

obturator is taken out and placed on the sterile tray. A dry 20-ml syringe is attached to the needle hub and forceful aspiration made. The patient experiences a pain at this time and should be forewarned about it so that he does not jump or become frightened. As soon as bloody fluid appears, the needle, still attached to the syringe, is withdrawn. The amount of material aspirated should not exceed 0.5 ml. After the removal of the needle from the puncture site, a sterile gauze square is firmly pressed against the wound while the smears are made, after which a band-aid or sterile compress is applied.

The operator, using the syringe as a control, and with needle attached, places very small drops of marrow material on coverslips or slides. Spreads of these drops are made by the technical assistant, and preferably by a person skilled in making smears properly. One of the most common errors in bone marrow procedures is the use of such big drops of blood that the preparations are too thick to examine. The average doctor has forgotten how to make good smears and should ask for help. Four to six smears should be made, after which it is recommended that several drops of marrow be placed on a slide, that the slide be tilted allowing the more liquid portion to run down and leaving bone marrow aggregates or bits of tissue. The excess blood is aspirated back into the syringe. A "squash preparation" is made of the tissue fragments by placing a slide firmly down on top of the particles and drawing the two slides apart. If desired, several drops of blood can be placed on a slide or in a tube containing dried oxalate or heparin, from which cell counts, reticulocyte counts and other procedures can be performed. Counts of bone marrow are varied due to uncontrollable dilution with peripheral blood and are of so little value in interpretation that this step in the procedure is not recommended.

Another elective procedure is the sectioning of the remaining clot. The marrow material left in the syringe is allowed to coagulate in the syringe, the barrel of which has been partially withdrawn to prevent sticking. This narrow cylinder or button of clotted blood and marrow is placed in 10 per cent formaldehyde or other fixative and held for tissue sections if desired. This combination of biopsy with aspiration is often of value and is particularly useful in thrombocytopenic purpura when there is doubt about the number of megakaryocytes, in granuloma and malignancy and in osteosclerotic lesions. The marrow and blood films are stained with Wright's or equivalent stain. The staining time is longer for marrow than for peripheral blood. The less perfect slides should be saved for a peroxidase stain, which is helpful in making decisions when the cells are of undifferentiated types.

Heparinization and Centrifugation (Limarzi). In this technique, approximately 1 ml. of blood is aspirated and placed in a corked paraffin-lined tube containing a minute amount of powdered heparin, which has been prepared especially for cytological studies (Hynson, Wescott and Dunning, Baltimore, Md.). The tube is shaken in order to mix. The marrow is pipetted into a Wintrobe hematocrit tube and centrifuged for approximately 5 minutes at 2,000 r.p.m. Centrifugation causes the material to separate out into a layer of fat which floats at the top, a layer of plasma, a buffy coat predominantly made up of nucleated cells and a layer at the bottom consisting of non-nucleated red cells. The height of the various layers is noted and recorded, after which the fatty layer and most of the plasma layer is aspirated and discarded. The myelocytic-erythrocytic layer, together with a small amount of plasma, is pipetted and transferred to a paraffin-lined watch glass, where it is mixed. Spreads are made on slides. The smears are stained with Wright's in the usual way.

The method gives some quantitative measure of the relative amounts of fat, plasma and cells in the bone marrow, but there is poor correlation with biopsy sections. The average normal values for the various layers are 3.2% for the fat, 45% for the plasma, 6.8% for the myelocytic-erythrocytic layer and 45% for the erythrocytes. One advantage of the method is that a larger number of nucleated cells can be seen in a given oil immersion field. The method is the procedure of choice when lupus erythematosus is suspected and "L. E." cells are to be looked for.

One disadvantage of the method is that the paraffin tube and watch glass have to be prepared, more equipment is needed, and more time is required of both the operator and the technical assistant. Degenerative changes and vacuoles appear in the cells during the process of heparinization, centrifugation, mixing and exposure to air and glass surfaces, making it more difficult to perform accurate differential counts. Although the method is often called a concentration method, it is really a method which dilutes the bone marrow with peripheral blood and then concentrates the normal white cells along with the myeloid cells.

Bone Marrow Biopsy

Bone marrow biopsy or trephination of bone, which consists in the removal of blocks or bits of tissue from the marrow cavity, eliminates the error due to admixture with circulating blood, and yields material which reveals the degree of hyperplasia and hypoplasia, the relative amounts of marrow, fat and bone, abnormalities in bone architecture and the presence of malignant cells. Marrow biopsy is indicated as a second

procedure after unsuccessful aspiration and in conditions in which malignancy, osteosclerosis or aplasia are suspected. The disadvantages of marrow biopsy are that it is a surgical procedure and leaves a disfiguring scar.

Equipment. Equipment for bone marrow biopsy can be assembled on a tray and transported to the bedside, but it is more convenient to schedule the procedure in the operating room where facilities for scrubbing up, preparing the operative field and draping of the patient are available. In addition to surgical tools for making the incision, controlling bleeding and suturing, the following special equipment is needed:

Mastoid retractors
Periosteal elevator
Cork borer, $\frac{3}{8}$ inch in diameter
Bone curette or spoon spatula

Procedure. The patient is prepared for operation by administration of an analgesic such as morphine. The skin over the selected site is cleaned with the usual surgical antiseptics, draped with towels and the local anesthetic such as 1% procaine injected. After a few minutes a skin incision, 1 to 2 inches long is made. Retractors are used to separate the edges of the skin. The periosteum is cut and scraped from the underlying bone in an area 1 to 2 cm. in diameter.

At this point it is recommended that a marrow puncture be performed with a standard marrow needle and that marrow for smears and culture be collected, for it is difficult, after the cavity has been opened, to prevent the inflow of peripheral blood or to aspirate representative marrow cells. By combining aspiration with culture and biopsy maximal information is obtained.

A window is now cut through the cortex, using an ordinary cork borer and the plug of cortex with its attached trabecular marrow removed and placed in formaldehyde or other fixative preferred by the pathologist who is to examine the specimen. A trephine or knife point may be used to cut out the desired block of tissue. Following this the marrow cavity is scraped with a bone curette and pieces of marrow scraped out. These are placed on a piece of gauze and handed out of the operative field to an assistant who makes several imprint preparations by pressing the surface of a slide directly down on the fragments of bone marrow and lifting the slide straight up, leaving a thin deposit of cells adhering to the glass. The remaining portions of bone marrow are placed in the fixative. After decalcification the blocks of tissue are sectioned and stained with hematoxylin and eosin or special stains.

The trephination of bone can be accomplished without making a skin incision by using the Turkel trephine instrument (Trephine Instruments Co., Detroit, Michigan), which consists of a sharp needle which is passed to the periosteum and serves as a guide for an inner trephine. The trephine cuts out a small core of bone and marrow which can be sectioned and from which imprints or smears can be made. Following the removal of the trephine, the outer needle can be pushed into the hole and marrow aspirated or fluids infused.

The examination of marrow sections should include an appraisal of the relative amounts of fat, connective tissue, bone and marrow cells, the notation of abnormalities in bone structure, vascular pattern, the presence of abnormal or malignant cells, the distribution of recognizable cell types, the predominant cells and presence of necrosis, hemorrhage or inflammatory reaction.

Examination

The first steps in the examination of smears of bone marrow are to look at the preparations under low power to see if the stain is satisfactory, to pick out the best smears and fields for more detailed study under oil immersion and to get an overall idea about the cell distribution, the amount of fat, the number of megakaryocytes and the presence of tissue fragments or nests of malignant cells.

It is recommended that a differential count of at least 200 nucleated cells be made and reported, for a differential count, while subject to considerable variation due to uneven distribution of cells and errors caused by a relatively small sample, furnishes objective data of value in interpretation and in following progress and response to therapy. A cell by cell study of several hundred cells and the placing of each cell in its appropriate category forces the examiner to observe the cells critically. A suggested form of marrow report with the range of normal values for the more common cells is given in Table 2.4. Cells such as megakaryocytes, macrophages and tissue basophils are not included in the myelogram, for these cells occur in such small numbers that their numerical evaluation has little significance.

Many hematologists do not recommend the performance of or the reporting of differential counts of marrow smears and consider that the procedure takes too much time, is inaccurate and is not necessary. It is conceded that the procedure is time consuming and that experts who have studied and counted cells for many years have acquired skill and judgment in evaluation and interpretation and that for such rare in-

TABLE 2.4

EXAMINATION OF BONE MARROW

Date.....

Name..... Age..... M F W

Site..... Amount Aspirated..... Operator.....

Red Blood Cells

Megakaryocytes Thrombocytes

Nucleated Cells. Number Counted..... Remarks

Normal		Per Cent
0–1%	Stem Cell	
0–1	Myeloblast	
1–5	Progranulocyte	
2–10	N. Myelocyte	
5–15	N. Metamyelocyte	
10–40	N. Band	
10–30	N. Segmented	
0–3	Eosinophil	
0–1	Basophil	
5–15	Lymphocyte	
0–1	Plasmocyte	
0–1	Histiocyte	
0–2	Monocyte	
	Atypical cell	
0–1	Rubriblast	
1–4	Prorubricyte	
5–10	Rubricyte	
10–20	Metarubricyte	
4:1	WBC: Nucleated RBC Ratio	

Summary of Abnormalities

Interpretation

Examined by.....

dividuals the differential count is superfluous. For the average examiner, for the sake of the records and for the training of junior staff, house officers and technicians there is merit in the study of individual cells. Inexperienced examiners are likely to be unduly impressed by

large and darkly stained cells, or by cells such as eosinophils or plasmocytes which they are looking for to establish a suspected diagnosis and are likely to have poor judgment concerning the relative numbers of cells. It is not fair to the patient for the examiner to merely state his opinion and not give the evidence upon which that opinion is based, for the interpretation of the bone marrow is no longer the prerogative of a chosen few. A study of many fields and several slides is more important than counting a few hundred cells and not looking at the rest of the smear, but a thorough overall examination plus a differential count yields maximal information.

Marrow examinations are now so widely used that it is necessary in many hospitals and clinics to train technicians to assist in the differential counts, so that the physician who is responsible for the examination and report does not have to perform the actual counts personally.

The number of megakaryocytes is determined by searching all parts of the various smears under low power, for megakaryocytes are characteristically unevenly distributed and often are found in clumps of cells or at the ends of the smears. If there is thrombocytopenia, at least 25 megakaryocytes should be studied to see whether or not they are producing granular platelets.

Search should be made for islands of cells suggestive of malignancy or unusual cell groups. In unexplained fever and splenomegaly one should look for etiologic agents such as histoplasma.

A peripheral blood smear should be available at the time of marrow examination in order that the two can be correlated. The morphology of erythrocytes can be best studied in blood smears. The distribution of thrombocytes is better and evaluation of their number more accurate in peripheral blood smears than in bone marrow preparations.

Analysis of Abnormalities

After the marrow and the peripheral blood have been examined, the differential recorded and a description made of unusual morphological variations in cells, the next step is to analyze the abnormalities to determine the state of the bone marrow and if possible to make a diagnosis.

From the smear alone it is not possible to tell whether the bone marrow is abnormally cellular or hypocellular. If there are many nucleated red cells in every oil immersion field, little fat and many large clumps of tightly packed nucleated cells, one is justified in stating that the marrow is cellular, but the degree of cellularity cannot be accurately stated without a section of tissue. On the other hand, if the marrow cells are scattered, if there are few nucleated cells and the differential count of

the bone marrow and the peripheral blood are similar, one cannot tell whether the marrow is hypoplastic or diluted with peripheral blood. If there was difficulty in aspirating fluid and dry taps on previous attempts, one is justified in assuming that the marrow is relatively aplastic or replaced, but biopsy is necessary to confirm this conclusion. Inability to control or to appraise the amount of dilution with peripheral blood is one of the most important handicaps of marrow smears and a reason for combining sections of the clot with smears.

The next step in the procedure is to study the various cell groups in relation to each other. The normal white cell to nucleated red cell ratio is approximately 4:1, with a range of normal variation of 6:1 to 2:1. A ratio of less than 2:1 is indicative of erythrocytic hyperplasia, whereas a ratio greater than 6:1 suggests erythrocytic hypoplasia or leukocytic over-stimulation. In a similar manner one looks at the relative numbers of eosinophils, basophils, lymphocytes, plasmocytes, monocytes and histiocytes. Assuming that there is no significant dilution with peripheral blood an eosinophil count above 5, a lymphocyte count above 20 and a plasmocyte count above 5 are probably significant and should be given weight in interpretation. If the marrow is practically replaced by one cell type, this is indicative of leukemia. On the other hand an increase in white cells of varied types (eosinophils, plasmocytes, monocytes and histiocytes) is indicative of a chronic inflammatory reaction or granuloma and is against the diagnosis of leukemia.

After the major cell groups are evaluated in relation to each other, one looks in turn at the maturation sequence of each group. In the normal bone marrow the majority of neutrophils are at the band or segmenting state of development and the nucleated red cells are predominantly producing at the rubricyte-metarubricyte level. If there is an increase in immature forms this should be noted.

Degenerative changes, abnormalities in morphology, hypersegmentation, abnormal sizes, asynchronism in development of nucleus and cytoplasm and abnormal mitoses should also be recorded in the analysis.

Interpretation

The bone marrow examination is seldom diagnostic, but like other clinical and laboratory procedures, it constitutes a link in the chain of evidence which, when correlated with other information, is often of value in diagnosis.

For details concerning the marrow findings in various diseases, see Chapters 3, 4, and 5 under the headings of various entities.

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CHAPTER 3

ANEMIAS, ERYTHROCYTOSES, HEMOGLOBINURIAS AND ABNORMAL HEMOGLOBIN COMPOUNDS

By L. W. Diggs

ANEMIAS

Definitions

Anemia may be defined as a condition characterized by a decrease in the oxygen carrying capacity of the blood or a decrease in the hemoglobin value, the packed cell volume, or the erythrocyte count of more than two standard deviations below the mean normal determined by the same method on the bloods of healthy persons of the patient's age and sex group.

Nutritional Factors

Food factors essential for red cell formation and hemoglobin synthesis are proteins, carbohydrates, fats, minerals and vitamins in the proper proportions. Included in the more potent blood building foods are those with high protein, iron, and vitamin content such as liver, brain and other organ meats, meats, sea foods, egg yolk, dried legumes and molasses. Cereals, nuts and green leafy vegetables have intermediate value. Milk, fruit juices, root vegetables, and carbohydrates as a class have relatively poor blood building potentialities. Excess fats and oils, including mineral oil, and excess calcium may interfere with digestion and absorption.

Iron, an essential constituent of hemoglobin and of all body cells, is usually supplied in adequate amounts by the average diet which contains approximately 5 to 15 mg. per day. Less than 1 mg. of iron is lost in the urine, bile and other secretions per day. Iron that is released in the body when red cells are destroyed and the hemoglobin split into its constituent parts is retained in the body and reutilized in hemoglobin synthesis. Increased requirements for iron occur when there is increased demand as in blood loss, pregnancy, lactation and in periods of rapid growth. Women are more likely to have deficiency of iron because of menstrual blood loss. Iron is absorbed from the stomach and small intestine. Free HCl in the gastric juice and ascorbic acid favor absorption. The absorption of iron is greater in iron deficient states than under normal conditions. Faulty digestion and diseases of the gas-

trointestinal tract decrease absorption. Infection interferes with utilization. Approximately 60 per cent of the 4 to 5 gm. of iron contained in the body is present in the hemoglobin of the circulating cells and approximately 20 per cent is available in storage in the liver, spleen, bone marrow and other organs. The anemia which is caused by iron deficiency is of a microcytic hypochromic type.

In addition to iron, other minerals such as copper, cobalt and magnesium are essential, but are needed only in trace amounts and their lack seldom is the cause of deficiency states or anemia.

Proteins furnish amino acids which provide essential building material for the synthesis of hemoglobin and other proteins.

Vitamins of the B complex, including B_{12}, folic acid (pteryolglutamic acid, PGA) and its metabolically active product, citrovorum factor (CF, folinic acid), are essential for the synthesis of nuclear proteins and for the normal growth, maturation and maintenance of body cells. B_{12} and folic acid are thought to be coenzymes which play supplemental and interdependent roles in the manufacture of proteins, exerting their activity at different stages in the building of complex compounds from simple substances. Both are necessary and one is ineffective in the total absence of the other. Deficiency of B_{12} and folic acid leads to abnormalities in the structure of cells and defective maturation, producing a marrow in which megaloblasts (rubriblasts of the pernicious anemia type) predominate. A reduction in the rate of mitosis and longer growth periods between cell divisions cause the cells to be macrocytic. The giving of B_{12} and/or folic acid to patients with megaloblastic macrocytic anemias corrects the deficiency and causes the marrow to revert to a normal state.

B_{12}, which is the active principle of liver extract and the substance which by usage has come to be known as the "extrinsic factor," requires the presence of an additional factor, known as "the intrinsic factor" which facilitates intestinal absorption. The "intrinsic factor" is present in the mucoprotein fraction of gastric secretions produced in the fundus and body of the stomach. In patients with pernicious anemia and related conditions there is a lack of or deficiency of the intrinsic factor which prevents the absorption and utilization of the B_{12} present in foods. Parenteral injections of B_{12} or the oral administration of B_{12} mixed with normal gastric juice or concentrates of intrinsic factor correct the deficiency.

Folic acid exists in various foods in a conjugated form which requires alteration by digestive processes before it is available for use. The

metabolic pathway is thought to be:

Folic acid conjugate (PGA conjugate) $\xrightarrow{\text{conjugase}}$ folic acid (PGA)

$\xrightarrow{\text{ascorbic acid}}$ citrovorum factor (CF, folinic acid)

Folic acid and folinic acid do not require the intrinsic factor for their absorption and utilization. In diseases such as tropical macrocytic anemia, sprue, and the macrocytic anemia of pregnancy and infancy, in which there is no deficiency of intrinsic factor, folic acid corrects the deficiency. In pernicious anemia, on the other hand, folic acid does not produce a complete hematological remission nor prevent neurological degenerative changes, for in this disease intrinsic factor is missing and B_{12} is not available for hematopoiesis.

Ascorbic acid aids in the formation of metabolically active growth factors and their absorption from the gastrointestinal tract. Ascorbic acid also favors iron absorption. Vitamin D and other vitamins are essential to body economy and when deficient indirectly play a role in anemia production.

Nutritional deficiencies are likely to become manifest after long periods of malnutrition and during periods of stress and increased metabolic activity. Nutritional anemias are seldom due to a lack of a single food factor but to multiple deficiencies. Not only is it necessary to ingest foods of proper kinds in adequate amounts, but the foods must be properly prepared, digested, absorbed and utilized in the body.

Anatomical Factors

Defective dentures may play a role in the production of anemia by limiting types of food eaten or preventing utilization of ingested foods. Diseases of the salivary glands, stomach, duodenum, liver or pancreas which interfere with proper production of digestive ferments may cause anemia although food intake is that which would be adequate for a normal person. Partial obstruction, inflammation or ulceration of the intestine, short-circuiting operation or fistula, and abnormal motility of fecal contents may interfere with absorption of essential foods or may be associated with changes in microbial flora which interfere with essential enzyme systems.

The bone marrow itself may be replaced by inflammatory lesions, scar tissue, or tumor cells producing the so-called "myelophthistic anemias."

Diseases involving the lymphatic system, spleen, liver, endocrine glands and kidney are often associated with anemia. Because of the

importance of anatomical structures in blood formation and destruction, one should investigate all systems and not be content with examination of the blood alone.

METHODS USED IN DIAGNOSIS

History, Physical Examination, and Initial Laboratory Procedures

Since anemia is usually a manifestation of diseases involving various systems of the body, the first approach to diagnosis is by means of the history, physical examination and screening laboratory procedures. In taking the history, special inquiry should be made concerning other members of the family, occupation, exposure to chemicals, medications, food intake, digestive disturbances, blood loss and previous laboratory findings. Physical examination should include retinal and rectal examination and testing of reflexes and vibration sense. Special attention should be given to skin color, jaundice, vascular changes, petechiae and ecchymoses, the condition of the tongue, lymph nodes, liver and spleen. Initial laboratory procedures should include the blood smear examination, hematocrit and sedimentation rate and urinalysis.

Mean Corpuscular Values

Having determined that a state of anemia exists the next step is to type the anemia by performing a red cell count, hemoglobin and packed cell volume, and by calculating the mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration (see Chapter 1). Blood smears and a reticulocyte count should be made at the same time.

If the anemia is microcytic and hypochromic, the most likely possibilities are blood loss, nutritional iron deficiency or hereditary leptocytosis. If the anemia is macrocytic, pernicious anemia or related diseases are suggested (Table 3.1).

Estimation of the size and hemoglobin content of red cells is of value in directing treatment. In microcytic hypochromic anemia iron therapy is indicated. Vitamin B_{12} (or liver extract) and/or folic acid are indicated in macrocytic anemias. The majority of anemias are normocytic normochromic and are not likely to be benefited by specific antianemic drugs.

Erythrocyte Formation, Loss and Destruction

Red blood cells are being continually formed and continually lost and destroyed. The level of the various cellular constituents in any given

TABLE 3.1
Microcytic Hypochromic Anemia

Chronic blood loss
Defective intake, absorption or metabolism of iron
Simple achlorhydric anemia
Hereditary leptocytosis

Macrocytic Anemia

Pernicious anemia
Megaloblastic macrocytic anemia (pernicious anemia type) due to or associated with:
Nutritional deficiency (tropical macrocytic anemia)
Infancy
Pregnancy
Tropical sprue
Non-tropical sprue (idiopathic steatorrhea)
Pellagra
Gastric lesions, including gastrectomy, carcinoma, polyp
Intestinal lesions, including partial obstruction, short circuiting operation, fistula, chronic infection, celiac disease
Diphyllobothriasis and other intestinal parasitic diseases
Megaloblastic macrocytic anemia of unknown cause (achrestic anemia, refractory macrocytic anemia)
Non-megaloblastic macrocytic anemia:
Diseases characterized by increased number of reticulocytes, including hemolytic anemias and recovery from marrow aplasia
Hypoplastic anemias
Myeloproliferative diseases
Malignancies involving the bone marrow (myelophthisic anemias)
Hypothyroidism (myxedema)

specimen of blood is determined by the balance that exists between formation on one hand, and loss and destruction on the other. In evaluating an anemia one should perform appropriate tests to detect abnormalities in each of these factors (Table 3.2).

Erythrocyte Formation. Variations in size, shape, color and hemoglobin content of erythrocytes and stippled cells in the stained blood smear are presumptive evidences of defective red cell formation. An absence of diffusely basophilic cells or of nucleated red cells in the stained smear and a low reticulocyte count in the presence of a severe anemia are indicative of hypocellularity or of defective delivery mechanisms.

Numerous nucleated red cells in the peripheral blood and a high reticulocyte count are indicative of abnormal marrow activity or extra-

TABLE 3.2

Laboratory Tests Used in the Diagnosis of Anemias

Degree of anemia	Packed cell volume (hematocrit) Hemoglobin Red cell count Total blood volume and red cell mass
Size and hemoglobin content of erythrocytes	Blood smear Mean corpuscular values
Defective blood cell formation	Blood smear Reticulocyte count Bone marrow
Blood loss	Feces for occult blood
Increased red cell destruction	Serum bilirubin or icteric index qualitative van den Bergh or 1-minute bilirubin test Blood smear red cell size, shape, structure, etc. immature red cells diffusely basophilic and stippled red cells appraisal of platelet number Reticulocyte count Leukocyte count Bone marrow examination Urine urobilinogen and bilirubin hemoglobin hemosiderin Feces urobilinogen
State of the bone marrow	Bone marrow aspiration or biopsy

medullary hematopoiesis, but are not necessarily indicative of effective red cell production. A rising red cell count, hemoglobin and packed cell volume with few or no nucleated red cells are the signs of effective regeneration.

Bone marrow aspiration and biopsy yield direct evidence concerning the state of the bone marrow. Roentgenograms of bone may reveal abnormality if there is pathological calcification or bone destruction,

but may not show changes in hyperplastic conditions or hypoplastic states in which there is replacement of the marrow cells by fat, fibrous tissue, or tumor cells.

Blood Loss. In addition to routine questioning of the patient for blood loss one should examine the stool for occult blood whenever there is an unexplained anemia, and particularly when the anemia is microcytic and hypochromic.

If the patient is being sent for diagnostic study and it is known that there is anemia that has not responded to the usual medications, he should be instructed to bring a specimen of feces for examination as a part of the initial survey. If a specimen is not available, a portion of feces sufficient for occult blood and microscopic examination can be obtained readily on the gloved finger at the time of the routine rectal examination. If the occult blood test is positive, drugs should be discontinued and the patient placed on a meat-free diet for several days, after which the stool examination is repeated.

Excessive Red Cell Destruction. In diseases characterized by increased red cell destruction the primary defect may reside in the red cells themselves, in the plasma, or in the increased phagocytic activity of the macrophage system. Red cells with structural defects, as in hereditary hemolytic anemia, do not survive for the usual 120 days in the circulation of the patient or when transfused into a normal individual. In diseases such as malaria or Oroya fever, the red cells are invaded by organisms which destroy the parasitized cells. Certain animal and plant poisons, numerous chemicals, toxins associated with bacteremias, and excessive heat or thawing after freezing cause erythrocyte injury. Hemolytic processes may be caused by hemolysins such as anti-A and anti-D, or by hemolysins developed as a result of antigens such as tumors, necrotic tissue, inhalants, foods, chemicals, parasites, bacteria, or fungi.

In conditions characterized by splenomegaly, there is an increase in the total mass of splenic pulp in which the red cells are sequestered with attendant increased red cell destruction. Spherocytes and other deformed cells are thought to be retained and destroyed in the splenic pulp. Senile cells as a class are more susceptible to hemolytic and phagocytic mechanisms than are normal cells.

Factors such as stasis, hypoxia, increased acidity of the blood, erythrocyte agglutination, and spherocytosis favor hemolysis. Any condition, whether local or systemic, which slows down the flow of blood is associated with an increase in carbon dioxide, a decrease in oxygen, and an increase in volume of individual red cells with a change in shape from

biconcave disc to spherical (Fig. 3.1). Any factor which favors agglutination creates clumps of cells which circulate less readily than do free cells through capillaries. Blood in thrombosed vessels, in infarcted areas, in serous cavities or extravasated in tissues undergoes destruction with ultimate phagocytosis of cells and cell fragments.

Various laboratory tests are used to detect hemolysis. A granular appearance of the blood suggests agglutination and potential hemolysis. Reddish discoloration of the supernatant fluid after red cells settle out or are separated by centrifugation is indicative of intravascular hemolysis. Incubation of the blood for one or more hours at 37°C. or exposure

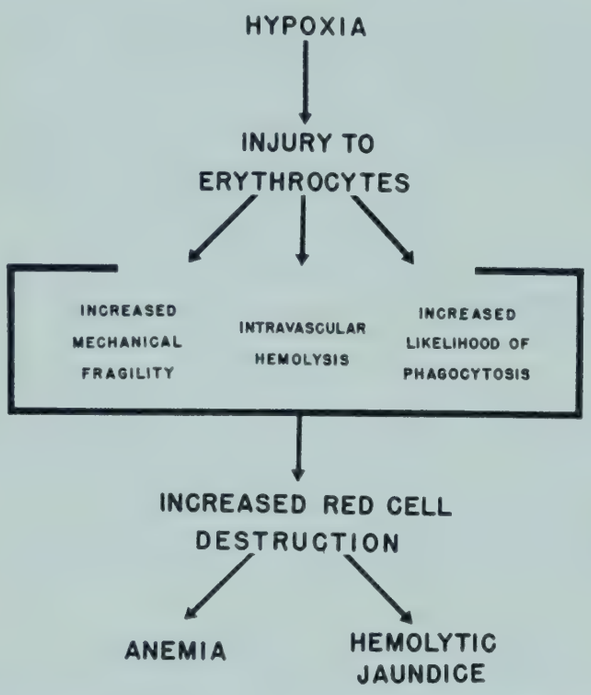


FIG. 3.1. The factors involved in production of anemia and hemolytic jaundice as a result of hypoxia.

of the blood to carbon dioxide or the addition of acids with slight reduction in the pH may reveal hemolysis which is not otherwise manifest. Other abnormalities are detected by noting agglutination of red cells in the cold (cold agglutination test) and hemolysis which occurs after chilling and subsequent warming (Donath-Landsteiner test). The osmotic fragility test is used to detect cells which have a reduced resistance to hypotonic salt solutions. Blood can be agitated with glass beads and the rate of blood destruction noted (mechanical fragility). In certain hemolytic processes the antibodies are adsorbed on the red cell membrane but hemolysis or agglutination may not be obvious. Sensitized or antibody-coated red cells will agglutinate when mixed with rabbit serum which contains antibodies against human globulins which have been developed in the rabbit by injections of human blood. (See Chapter 6, Coombs test.)

TABLE 3.3

Special Tests for Specific Diseases

Sickle cell disease	Sealed moist preparation Blood plus reducing agents Paper electrophoresis
Hemoglobin C	Paper electrophoresis
Spherocytosis	Osmotic fragility Blood smear Moist preparation
Nocturnal paroxysmal hemoglobinuria	Acid hemolysis test
Paroxysmal cold hemoglobinuria	Donath-Landsteiner tests Serologic test for syphilis
Hemolytic diseases due to hemolysins (erythrocyte bound antibody)	Coombs test using immune rabbit serum Agglutination and lysis of incubated red cells
Lead poisoning	Basophilic aggregation count
Pernicious anemia and simple achlorhydric anemia	Gastric analysis

The spherical nature of red cells can be detected by examination of the ordinary blood smear. Spherical cells are evenly and darkly stained, lack the central achromia seen in normal cells and have a reduced diameter. An increased thickness of cells can best be demonstrated when they are in rouleaux formation in moist preparations.

In hemolytic anemias there is usually active compensatory blood regeneration manifested by hyperplasia of the bone marrow with a relative and absolute increase in nucleated red cells, a diminution in fat and extension of the red marrow into the shafts of the long bones. Nucleated red cells are likely to be present in the peripheral blood and the reticulocyte count is significantly increased. The red cells are likely to be abnormal in size and shape. Leukocytosis and thrombocytosis are the rule in hemolytic states, but hemolysis may occur in conditions which are characterized by leukopenia and thrombocytopenia.

The hemoglobin present in the phagocytized red cells is ultimately split into globin (a protein), iron, and bilirubin. The bilirubin in the

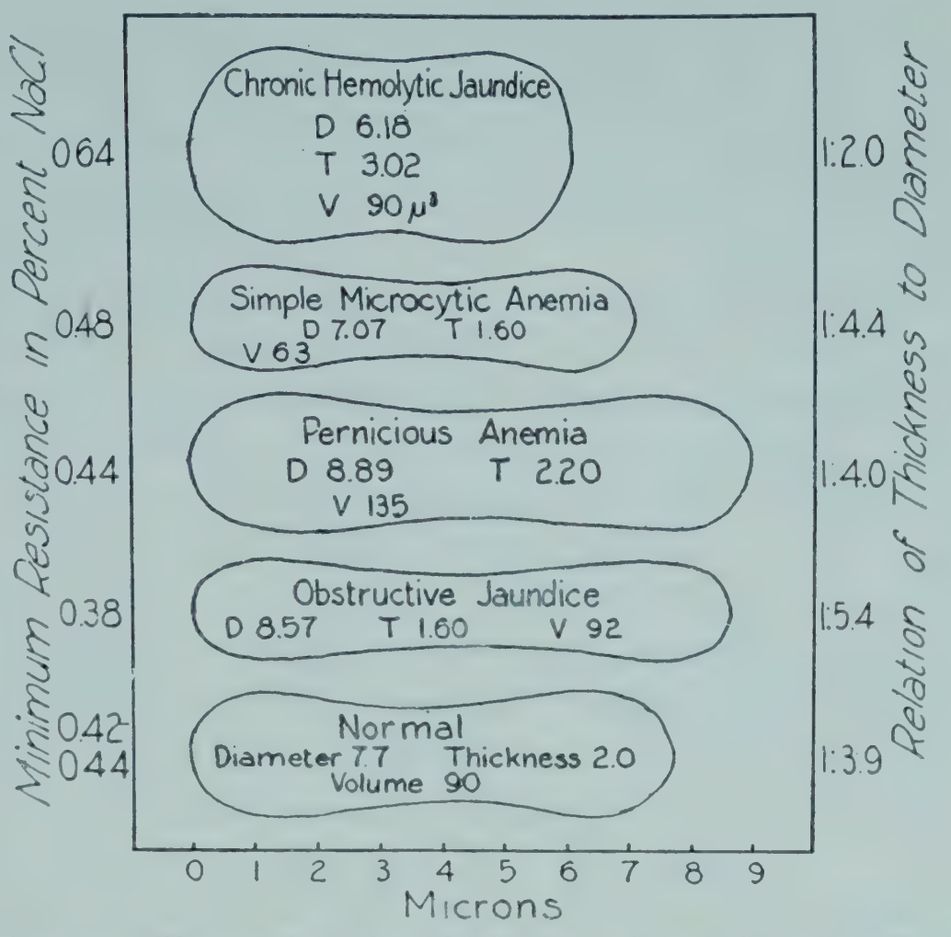


Fig. 3.2. Diagram illustrating the thickness of red blood cells in various types of anemia. Note the small diameter and increased thickness of the cell of chronic hemolytic jaundice, the normal thickness and increased diameter of the cells of pernicious anemia, the decreased thickness and decreased diameter of the cell of microcytic anemia. (After Haden: J. Lab. & Clin. Med., Mar., 1935.)

plasma gives to the plasma its characteristic yellow color. In conditions in which there is excess red cell destruction there is an increase in pigment production. When the amount of pigment produced exceeds the ability of the liver to excrete, jaundice is produced (Chapter 8, Liver Function). In hemolytic anemias in which there is no significant impairment of liver function or obstructive hepatic lesions the one-minute bilirubin is usually less than 0.5 mg./100 ml. The total bilirubin in uncomplicated hemolytic processes is elevated over the normal value of 1 mg./100 ml. but is usually less than 6 mg./100 ml.

It is possible to have increased red cell destruction without significant jaundice or anemia provided the liver is capable of excreting the excess pigment as fast as it is formed and the bone marrow is capable of regenerating cells as fast as they are destroyed. On the other hand, one may have rather marked jaundice and little anemia, or marked anemia and little jaundice, depending on the balances between formation and regeneration of cells and production and excretion of pigment.

In hemolytic processes there is more pigment produced than normal, more excreted by the liver and therefore more in the feces. In con-

ditions in which there is increased destruction of red cells the stools are darker than normal and the total quantity of fecal urobilinogen more than 300 mg./24 hours. There may be a significant increase in the quantity of urobilinogen in the stool without an increase in serum bilirubin.

Bilirubin which is excreted by the liver is reduced in the intestinal tract to urobilinogen, part of which is absorbed into the circulating blood and re-excreted by the liver with small amounts appearing in the urine. When urobilinogen is absorbed in excess quantities or the liver function is impaired, there is a significant increase in urinary urobilinogen. Values above 5 Ehrlich units of urine urobilinogen in 24 hours are indicative of increased red cell destruction and/or impaired liver function. In hemolytic processes in which the liver parenchyma is not involved in severe degenerative changes there is no obstruction of bile ducts or regurgitation of bilirubin and bile salts into the circulation and therefore an absence of bilirubin and bile salts in the urine (acholuric jaundice).

Normal blood plasma contains less than 5 mg. of hemoglobin and heme compounds per 100 ml. In conditions in which there is rapid intravascular hemolysis or excessive destruction of blood in extravascular areas as in crush injuries, infarcts or massive hemorrhages into tissues or serous cavities there is an increase in hemoglobin and heme-containing pigments causing the serum or plasma to be reddish-brown. The hemoglobin is excreted by the glomeruli of the kidney and reabsorbed by the tubule cells. Hemoglobin does not appear in the urine until the capacity of the tubule cells to reabsorb is exceeded. Hemoglobinuria is usually demonstrable when the concentration of hemoglobin in the blood plasma reaches levels of 100 to 150 mg. per 100 ml. Once hemoglobin appears in the urine it continues to be demonstrable until the blood plasma level decreases to 25 to 50 mg./100 ml. Part of the hemoglobin filtered by the glomeruli in the kidney is converted into hemosiderin and appears in the urine as yellowish-brown granules in epithelial cells or as free granules which can be identified by mixture of the sediment with aqueous ammonium sulfide which causes the hemosiderin particles to turn black. Urine which contains hemoglobin is dark red-brown to black and gives a positive benzidine test. Hemoglobin and other pigments can be identified by spectroscopic methods. Care must be taken to examine fresh specimens of urine, for red cells in the urine (hematuria) may be laked and give "false hemoglobinuria." Hematuria and hemoglobinuria may appear together, in which case the examination of the

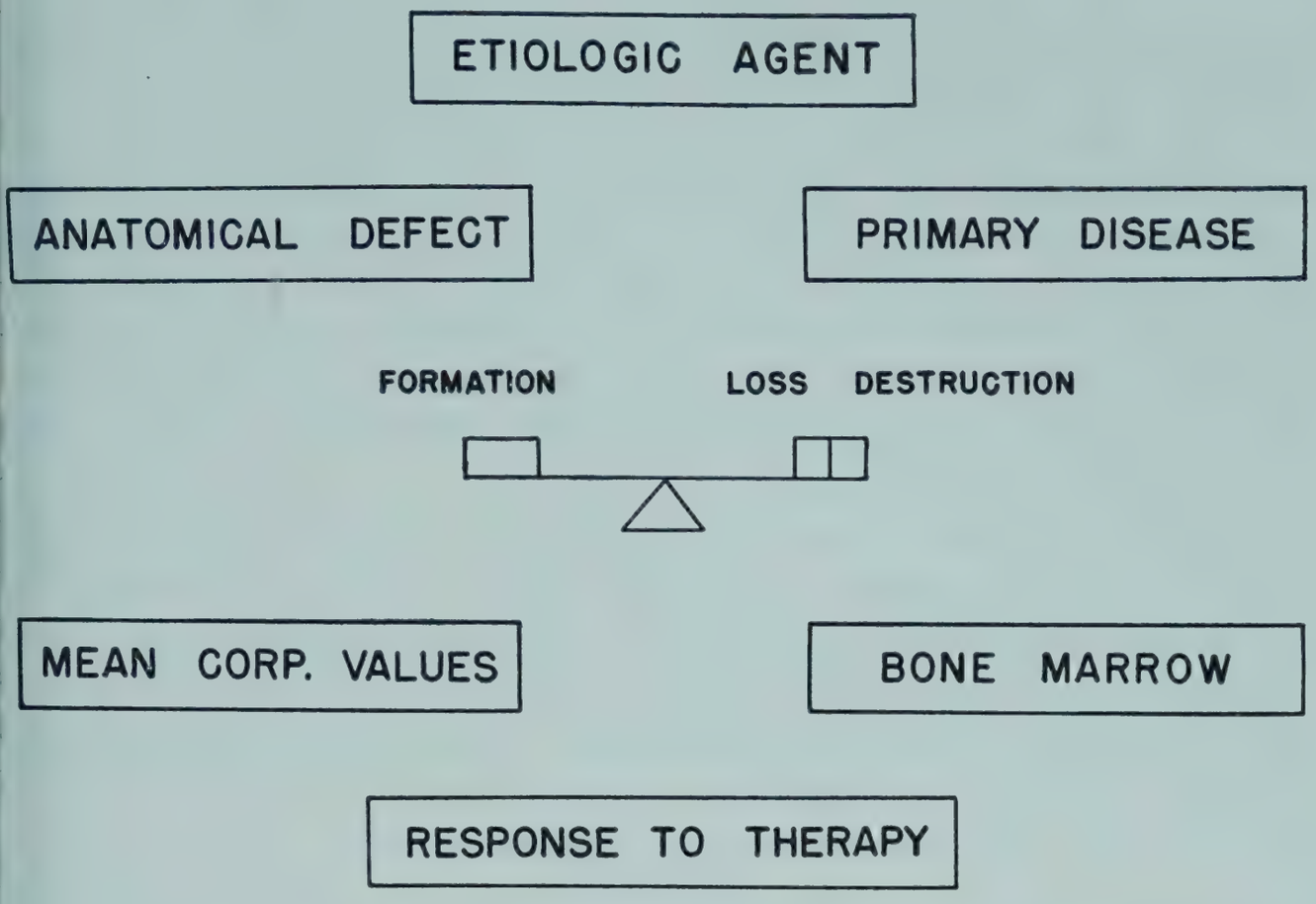


Fig. 3.3. Clinical approach to diagnosis

blood plasma for hemoglobinemia is essential in order to prove that there is excessive systemic red cell destruction. Following hemoglobinemia and hemoglobinuria there are varying degrees of jaundice and anemia.

Clinically, excessive hemolysis of red cells is characterized by such symptoms as chills and fever, nausea and vomiting, diarrhea, constrictive sensations in the chest, and yawning. Backache, headache and abdominal cramps are common. There may be thrombotic and embolic phenomena and skin rashes. In severe hemolytic processes there may be associated shock, anoxia and lower nephron nephrosis. Syndromes characterized by hemolysis of red cells and variants of the above symptoms are known as "hemolytic crises."

Pathologically, increased hemolysis due to any cause is characterized by splenomegaly, jaundice and by deposits of brown and black pigment granules in the phagocytic cells of the liver, spleen, lymph nodes and bone marrow and in the cells of the kidney tubules (hemosiderosis).

The Bone Marrow Response and the State of the Bone Marrow

The reticulocyte count, examination of the blood smear for immature red cells and examination of smears of bone marrow yield information

concerning the ability of the marrow to respond to anoxia caused by the anemia. The bone marrow study also reveals information concerning abnormal cells in diseases such as plasma cell myeloma, leukemia, metastatic malignancy, lupus erythematosus, and histoplasmosis.

Course and Response to Therapy

In many conditions one may not be able to make a diagnosis at the first examination, but by following the patient and noting the changes that take place on successive examinations, trends can be established and the true picture revealed.

Response to therapy may be a useful tool in diagnosis. If the diagnosis of pernicious anemia is made but the patient does not respond to adequate doses of liver, the diagnosis is incorrect. Iron deficient states should respond to iron, and anemias associated with scurvy to vitamin C. The anemia thought to be due to blood loss should improve promptly after the bleeding has ceased.

Summary

In order to make the final diagnosis and to classify the anemia the examiner should have obtained by history, physical examination and laboratory procedures information which would enable him to answer the following questions:

1. What is the etiologic agent?
2. Where is the anatomical lesion?
3. What is the primary disease?
4. What is the size and hemoglobin content of the average cell?
5. What mechanisms are primarily involved?
6. What is the state of the bone marrow?
7. What is the course of the anemia and its response to therapy? (Fig. 3.3)

CLASSIFICATION

The anatomical and etiologic classification given in *Standard Nomenclature of Disease*, published by the American Medical Association, is recommended for the classification and terminology of diseases characterized by anemia. Diseases that primarily involve blood forming organs are listed under rubric "*5*--" under the heading "Diseases of the Hemic and Lymphatic Systems." For filing purposes the first three digits designate the topographical site and the last three digits the etiologic agent.

PRINCIPAL DISEASES AND CONDITIONS CHARACTERIZED BY ANEMIA

Anemia Due to Nutritional Deficiency

Anemia Due to Defective Intake, Absorption, and Utilization of Iron (Nutritional Anemia Due to Iron Deficiency, Nutritional Microcytic Hypochromic Anemia). The most common cause of anemia is iron deficiency. Nutritional anemias of this type are seen in all age groups, in all walks of life and in association with all diseases. Included in the list of conditions in which there is microcytic hypochromic anemia in which benefit is to be expected following iron therapy are the anemia of prematurity, "milk anemia" due to milk ingestion with exclusion of other foods, anemia of childhood due to the increased demand of growth and anemia associated with pregnancy and lactation. So-called "chlorosis" is now thought to be a form of nutritional disease in young women who have inadequate food intake with superimposed menstrual blood loss. Many anemias are in part due to increased intestinal motility, excessive use of cathartics and mineral oils, and inflammation and ulceration of the large intestine.

In iron deficiency anemia the mean corpuscular volume is often below 70 cubic microns, the mean corpuscular hemoglobin less than 27 micromicrograms and the mean corpuscular hemoglobin concentration below 32 per cent. In many instances the red cell count may be normal or only slightly reduced although the hemoglobin is significantly decreased. Blood smears reveal variation in the size of red cells and an increase in the prominence of the central achromia (Fig. 3.4). Oval and elliptical cells, target cells and cells with irregular shapes are present. Polychromatic and stippled red blood cells and occasional nucleated red cells are demonstrable. The white cell count and cell distribution is essentially normal and the thrombocytes are adequate. There is no jaundice or evidence of increased red cell destruction.

The bone marrow is cellular with a relative increase in erythrocytic elements. Mitotic figures are common. The majority of nucleated red cells are in the rubricyte and metarubricyte stage. As a class the nucleated red cells are smaller than normal, are deficient in hemoglobin, have irregular shapes, frayed margins, pyknotic nuclei and cytoplasms that take a predominantly basic stain.

Iron in the form of ferrous sulfate or ferrous gluconate or other iron salts given by mouth will usually be followed by an increase in reticulocytes and increase in hemoglobin. B_{12} and liver extract are not indicated.

Simple Achlorhydric Anemia. One of the variants of iron deficiency anemia is a condition occurring most frequently in middle-aged women which is characterized by a lack of free hydrochloric acid in the gastric juice and a hypochromic microcytic anemia which responds to iron. Multiple epithelial changes which include premature graying of the hair, papillary atrophy of the tongue, esophageal mucosal changes with difficulty in swallowing (Plummer-Vinson syndrome) and brittle spoon-shaped finger nails are features of the disease. It is thought that the anemia is the result of a combination of factors including deficient diet, impairment of gastric function and blood loss (menstrual bleeding, hemorrhoids, etc.).

Pernicious Anemia. Pernicious anemia is a disease due to a deficiency of vitamin B_{12} occurring in adults, which is conditioned by a defect in gastric secretions and which results in defective red cell formation, a macrocytic type of anemia and a subacute combined degeneration of the spinal cord.

There is atrophy of the gastric mucosa, with absence of free hydrochloric acid after histamine stimulation. Papillae of the tongue are likewise atrophic. The tongue is red and often sore. The patients have a yellowish type of pallor and tissue changes associated with increased red cell destruction. The heart musculature is flabby and there are other parenchymal degenerative changes associated with severe anemia. Central nervous system degenerative changes are prominent producing symptoms and signs such as paresthesias, stiffness, impairment of position and vibration sense, ataxia, sphincter disturbances and varied reflex changes.

The bone marrow in pernicious anemia is cellular with replacement of the fat and extension of regenerative and red marrow into the shafts of the long bones. There is a relative and absolute increase in nucleated red cells with an increase in rubriblasts and prorubricytes. The majority of nucleated red cells are larger than normal (megaloblasts). In some cells nuclear chromatin is fine and delicate, in others coarse and irregularly pyknotic. Asynchronism between nuclear structure and cytoplasm is prominent with many early cells having abnormally large amounts of hemoglobin. Mitotic figures, abnormalities in nuclear division and multiple nuclear fragments are common. The neutrophils in all stages of maturation are large and have abnormal folds and lobulations. Maturation of these multilobulated and defectively formed neutrophils results in the macrocytic hyperlobulated neutrophils or "P.A. polys" (Fig. 3.5). Megakaryocytes are large and hyperlobulated and

thrombocyte production defective. Undifferentiated stem cells are conspicuous.

In spite of extensive marrow hyperplasia, production of red cells is inadequate to compensate for blood destruction. The cells, being defectively formed, have a shorter than normal survival time; and there is, as a result, jaundice of a hemolytic type with the usual laboratory findings (see under excessive red cell destruction).

The anemia is of a macrocytic hyperchromic type with mean corpuscular volume above 100 cubic microns and often in the range of 120–140 cubic microns. The mean corpuscular hemoglobin is usually above 33 micrograms. Although there is more than the usual amount of hemoglobin in the average cell, there is no increase in the concentration of hemoglobin per unit volume. There is more hemoglobin per cell because the cells are larger.

The stained smear reveals marked variations in size of red cells. There is variation in hemoglobin content and irregularities in distribution of hemoglobin within the cells. Poikilocytosis is marked in severe cases, with tear drop, pear-shaped cells and cells with blunt filaments predominant (Fig. 3.4). Nucleated red cells and cells with nuclear fragments are usually demonstrable in small numbers and macrorubriblasts (megaloblasts) may be found on searching. Cabot's rings and stippled cells may be present. In occasional smears there may be numerous nucleated red cells in all stages of development.

The reticulocyte count in untreated cases is usually 2 to 4 per cent. (See Chapter 1 for a discussion of the reticulocyte response following administration of vitamin B_{12} or liver extract.)

The white cell count in uncomplicated pernicious anemia is usually normal or below normal. There is, as a rule, a relative and absolute neutropenia with a relative lymphocytosis. Immature neutrophils and hyperlobulated forms are present. Thrombocytes tend to be decreased and there may be, in terminal stages, purpura, prolonged bleeding time, and defective clot retraction.

The above described clinical picture, pathologic changes and blood picture are those of severe and advanced pernicious anemia. There are many cases in which there is partial deficiency of vitamin B_{12} with mild macrocytic anemias and variants of the clinical features.

Subacute combined disease of the central nervous system associated with histamine achlorhydria may occur with no, or slight, anemia. In these cases there are usually a significant macrocytosis and bone marrow changes of the pernicious anemia type.

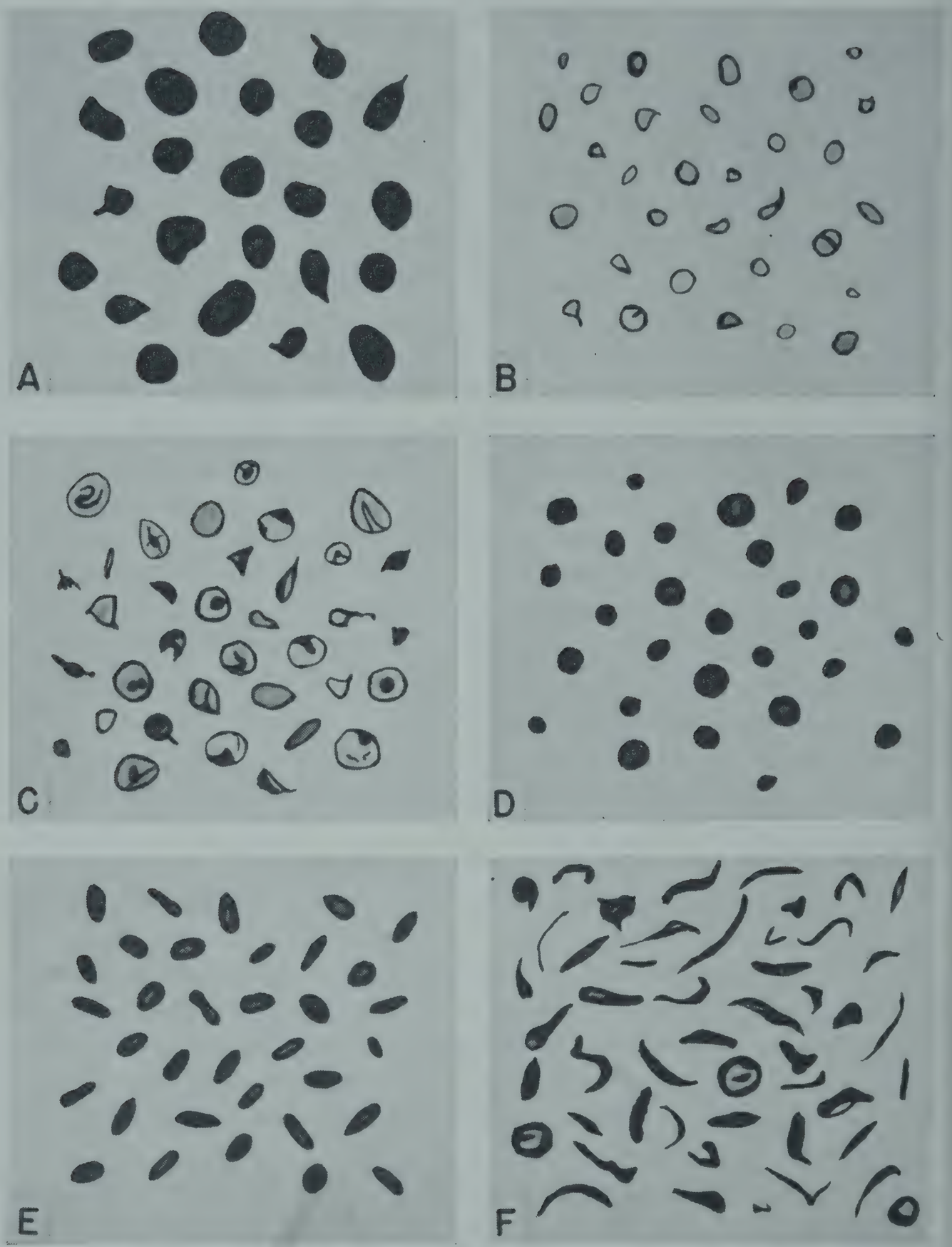


FIG. 3.4. Size and shape of erythrocytes in a) pernicious anemia; b) microcytic hypochromic anemia; c) hereditary leptocytosis; d) hereditary spherocytosis; e) hereditary ovalocytosis; f) sickle cell anemia.

Other Macrocytic Megaloblastic Anemias. In addition to typical pernicious anemia as described above, there are numerous other conditions or diseases which may be characterized by macrocytosis and bone marrow changes of the pernicious anemia or megaloblastic type (Table

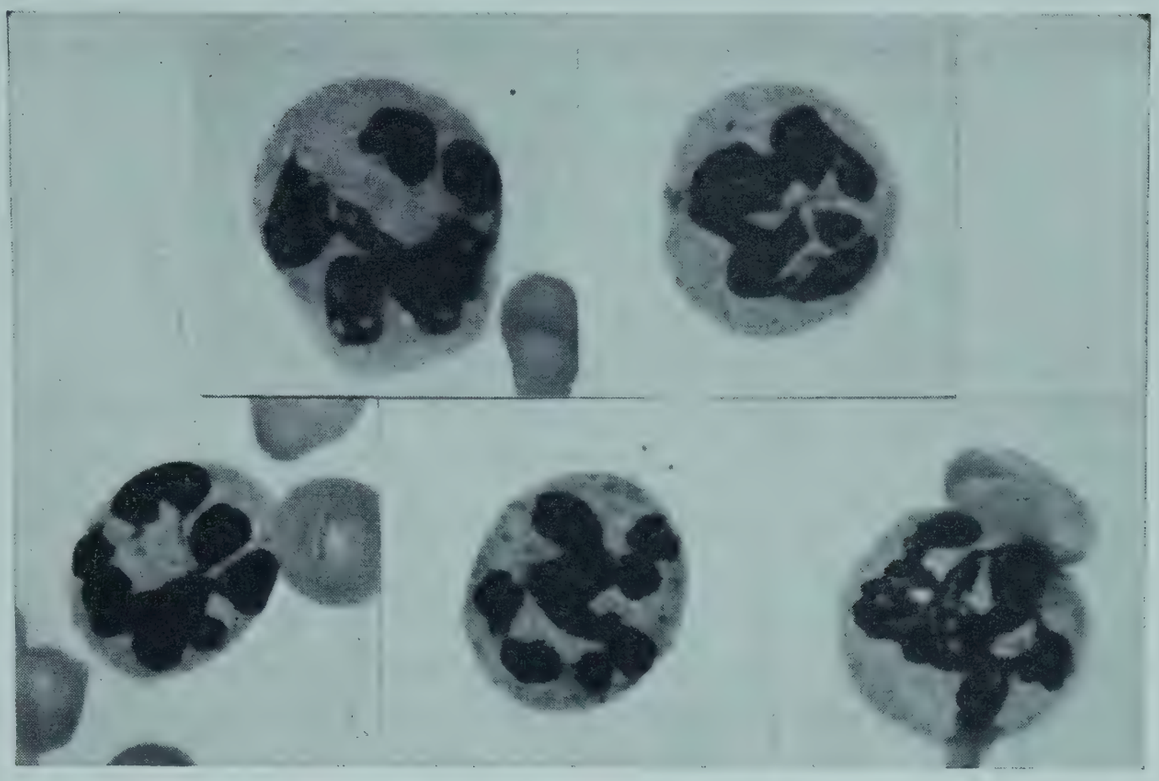


FIG. 3.5. Increased nuclear segmentation from a case of pernicious anemia. From Heck, F. J. and Watkins, C. H.: Am. J. Clin. Path. 3: July, 1933.

3.1). The common denominator in the majority of the diseases listed in Table 3.1 is a deficiency in the vitamin B_{12}-folic acid group of food factors which are necessary for formation of cellular proteins and for the maturation of megaloblasts.

As a class they differ from true pernicious anemia in that free hydrochloric acid are usually present in the gastric juice and central nervous system degenerative changes are usually absent.

Anemia Due to Blood Loss

Acute Blood Loss. Following acute blood loss there is a reduction in the total blood volume with no change in the erythrocyte values until fluid balance is restored and the circulating cells diluted. Shock states that often accompany blood loss tend to mask the anemia, for fluids escape from the blood vessels into tissue spaces and produce cellular hemoconcentration.

The first blood changes that are demonstrable following hemorrhage are an increase in thrombocytes and a decrease in the bleeding and coagulation time. Neutrophilic leukocytosis with a moderate left shift begins within a few hours. The total leukocyte count may be 20,000 to 30,000 per cu. mm. Red cell regeneration in the form of increased reticulocyte count begins on the day after hemorrhage and reaches its peak during the first week. Nucleated red cells are usually present in the stained blood smears in small numbers.

Following the loss of 500 ml. of blood, restoration in erythrocyte and hemoglobin values is usually achieved in 3 to 5 weeks. If 1,000 ml. is lost, there is slower recovery. Women as a class regenerate at a slower rate than men. Regeneration is favored by a high protein and iron diet. The maximal tolerated blood loss for the average adult is approximately 2,000 ml.

Chronic Blood Loss. The most common causes of chronic blood loss are excessive menstruation, hemorrhoids, and intestinal or gastric ulceration. Other causes include esophageal varices, tumors of the gastrointestinal, genital and urinary systems, polyps and telangiectasias.

In chronic blood loss the bone marrow reveals changes of the type described under nutritional iron deficiencies. The anemia is of a microcytic, hypochromic type with a slightly increased reticulocyte count and occasional nucleated red cells. Oval cells, target cells and variations in size and shape are demonstrable. Thrombocytes and leukocytes tend to be increased, with neutrophils increased more than other cell types. Serum bilirubin is decreased.

Anemia Due to Plant and Animal Parasite

Anemia is frequently associated with diseases of varied etiology. In chronic infections the anemia is usually normocytic and normochromic with feeble regenerative responses. In acute and severe infections there is often a leukemoid cellular reaction with nucleated red cells in the peripheral blood and evidence of increased red cell destruction. Infections may cause anemia by interfering with appetite, by causing faulty digestion and absorption of foods, or by interference in the synthesis of hemoglobin.

In infectious states the bone marrow may be hypoplastic and in terminal infections aplastic, but usually the marrow is cellular with vacuoles in cytoplasm or nuclei, pyknosis and increased prominence of basophilic granules. Stippled cells, anisocytosis and poikilocytosis are demonstrable. In most chronic infections there is an increase in plasmocytes, lymphocytes, monocytes and histiocytes. In pyogenic and coccal infections, neutrophilic leukocytosis with a moderate shift to the left is the rule. (See Chapter 5). Infections such as miliary tuberculosis or brucellosis may produce granuloma in the bone marrow.

In human bartonellosis and in plasmodium infections the red cells are invaded by organisms which cause blood destruction. In *Plasmodium falciparum* infections and occasionally in other types of malaria there may be rapid blood destruction with shock-like symptoms and hemo-

globinuria (blackwater fever). Hookworms and other intestinal parasites may produce anemia by causing blood loss or by utilization of essential food factors.

Because of the frequency of infections as a causative factor in anemia, one should always seek to identify the etiologic agent by history, examination of body fluids other than blood, and by cultural means.

Anemia Due to Intoxication

Anemia Due to Chemicals. We live in a chemical world and are continually exposed to chemical substances which have potential cytotoxic properties or which cause defective blood cell formation or hemolysis or interfere with essential enzyme systems. Occupational hazards include the use of fat solvents, cleaning solutions, paints and varnishes, coal tar products, inks and dyes, fumes of various kinds and heavy metals. Our vegetables and fruits are sprayed with poisons and we use insect repellants daily in our homes. Lotions and creams, deodorants, depilatories, permanent waving lotions, dentifrices, gargles, mouth washes, douches, contraceptives, headache remedies, sedatives, cathartics, salves, powders and drugs of infinite variety are prescribed or used without medical advice and often without thought concerning their harmful effects. Included in the more commonly used drugs which may cause anemia are the sulfonamides, gold salts, cinchophen, acetanilid and phenacetin, arsenic, mercury, bismuth compounds and benzedrine.

In any unexplained anemia, and particularly in those in which there are variations in size, shape and staining of red cells, stippled and nucleated red cells, one should go over the history carefully and attempt to ferret out possible chemical toxicity factors. Care must be taken in assuming that the anemia present is due to a given substance because there has been exposure. In the lead industry the finding of more than 10 stippled red blood cells in 50 oil immersion fields, or 2,500 red cells, is considered as significant of abnormal exposure to lead, but is not necessarily a sign of lead poisoning.

Space does not permit discussion of the type of anemia produced by each chemical and drug. The reader is referred to textbooks on toxicology and pharmacology.

Anemia Due to Animal or Food Poison. The venoms of certain snakes and insects may cause an acute hemolytic type of anemia. Certain plants such as mushrooms and other substances of vegetable origin may have cytotoxic effects. Plant and animal substances may serve as antigens for the development of specific hemolysins.

Anemia Due to Physical Agent

Mechanical trauma is a common cause of acute blood loss (see above). Excessive heat produces injury to erythrocytes with signs of hemolytic jaundice and in some patients hemoglobinuria. The anemia associated with extensive burns is thought to be due in part to the attendant infections.

Irradiation of any sort causes a suppression of mitosis, atypical mitotic figures and a decrease in the number of differentiated cells of all types. Patients receiving roentgen ray therapy, or being treated with radium or radioactive substances develop varying degrees of marrow hypoplasia with peripheral pancytopenias. Victims of heavy irradiation as in atomic bomb explosions have severe aplastic anemia with slow recovery. In the first weeks following exposure to radioactive substances, the predominant cells in the bone marrow are undifferentiated mesenchymal cells, plasmocytes and fat cells. Gradually granular cells, lymphocytes and erythrocytic islands appear. The marrow cellularity gradually increases. Hypercellularity may appear before there is significant improvement in the peripheral blood picture.

Physicians and attendants exposed to roentgen rays or radioactive elements should have periodic checks of their peripheral blood. Screening tests recommended are hematocrit, white cell count, reticulocyte count, and blood smear examination.

Anemia Due to New Growth

Anemia is often associated with neoplasms. In the lymphomas there is a shortened survival time of erythrocytes in the circulating blood and progressive anemia in spite of an increased rate of erythropoiesis. In acute and fulminating leukemias and in plasma cell myeloma there is a decreased rate of erythrocyte formation which in the presence of an increased rate of red cell destruction leads to a rapid decrease in erythrocyte values. Occasionally in malignant conditions and particularly in malignancies involving the mesenchymal cells there may be a severe hemolytic anemia of the antigen-antibody type. Other factors in the production of anemia associated with neoplasms may be inadequate intake of food, faulty digestion, poor absorption and blood loss.

The erythrocytic cells themselves may undergo neoplastic changes, producing the disease entity, erythroblastemia (erythremic myelosis, Di Guglielmo's disease). In this condition the normal bone marrow cells are replaced by malignant cells of the erythrocytic series, there are extramedullary islands of erythrogenesis and pathological nucleated

red cells in the peripheral blood. In some cases there may be a mixture of neoplastic erythrocytic cells with myeloid cells, producing a hematologic picture to which the name "leuko-erythroblastic leukemia" has been given. This latter condition furnishes another example of new growth which involves primitive mesenchymal cells and which does not adhere strictly to the differentiation of a single strain of definitive cells.

Anemia Due to Prenatal Influence, Hereditary and Familial Diseases

Sickle Cell Disease. The term sickle cell disease is used as a general term for any and all of the conditions and diseases characterized by the presence of hemoglobin S including the sickle cell trait without anemia, sickle cell anemia, and sickle cell hemoglobin in combination with other abnormal types of hemoglobin and with other hereditary diseases.

Sickle cell (S) hemoglobin is chemically and immunologically different from normal adult (A) hemoglobin and has a characteristic electrophoretic mobility. In the oxygenated state hemoglobin S has approximately the same solubility as hemoglobin A, but when deoxygenated (reduced) the solubility of hemoglobin S is significantly less than that of hemoglobin A, forming elongated crystal-like structures (tactoids) which distort the erythrocytes producing the characteristic "fish-fin," multipointed and biazarre forms known as sickle cells (Fig. 3.6). On reoxygenation the hemoglobin goes back into solution and the sickled erythrocytes promptly return to their original shape (Table 3.3).

The most practical methods used to demonstrate sickle cells are:

1. Sodium bisulfite method. A 2 per cent solution of sodium bisulfite in water is prepared. A large drop of this solution added to a small drop of blood causes a prompt sickling of erythrocytes. Tablets of sodium bisulfite, specially prepared for sickle cell testing are now available (A. S. Aloe and Company, St. Louis). One tablet dissolved in 10 ml. of distilled water gives the proper dilution. The solution should be made up fresh each day because it deteriorates rapidly on standing at room temperature.

2. "Moist stasis" preparation. A rubber band is placed around the proximal portion of a finger and is allowed to remain for approximately five minutes. The finger tip is punctured and a drop of blood is taken on a cover slip, immediately placed drop side down on a slide and the blood allowed to spread. Several preparations should be made. The margins are sealed with petroleum jelly. Preparations are examined microscopically. If no sickling is observed immediately, the preparations are examined again at the end of 24 hours. Sickled cells are charac-



FIG. 3.6 Sickle cells in sealed moist preparation.

terized by elongation and by multiple sharp points protruding from the margins of the cells (Fig. 3.6).

The red cells in sickle trait in the heterozygous form (S-A) contain a mixture of normal and sickle cell hemoglobin. Greater reduction in the oxygen tension is required to make these cells sickle than is necessary for the red cells in sickle cell anemia (S-S) which do not contain hemoglobin A. The crystallization of hemoglobin takes place more slowly and the degree of distortion is less marked in the sickle cell trait than in sickle cell anemia. The percentage of cells undergoing the change is not a distinguishing feature for in both conditions 100 per cent of the cells may undergo characteristic distortion if the proper chemical conditions are fulfilled.

Failure to find sickle cells in a given preparation does not rule out the presence of hemoglobin S for the amount of hemoglobin S may be too small to detect morphologically or the technique used may be faulty.

Common errors are improperly cleaned glassware, mixture of the blood with alcohol, trapping of air under the coverslip, insecure sealing, and use of an old solution of sodium bisulfite.

The long, pointed and relatively rigid sickled erythrocytes are associated with an increase in the viscosity of the blood and tend to produce blockage of blood vessels, stasis and hypoxia. The hypoxia causes injury to cells, vascular occlusive phenomena, tissue degenerative changes and necrosis (Fig. 3.7).

Sickle Cell Trait (Sickle Cell Trait without Anemia, S-A Disease, Heterozygous Sickle Cell Disease). The sickle cell trait occurs in approximately 8 per cent of the Negroes in the United States. There is

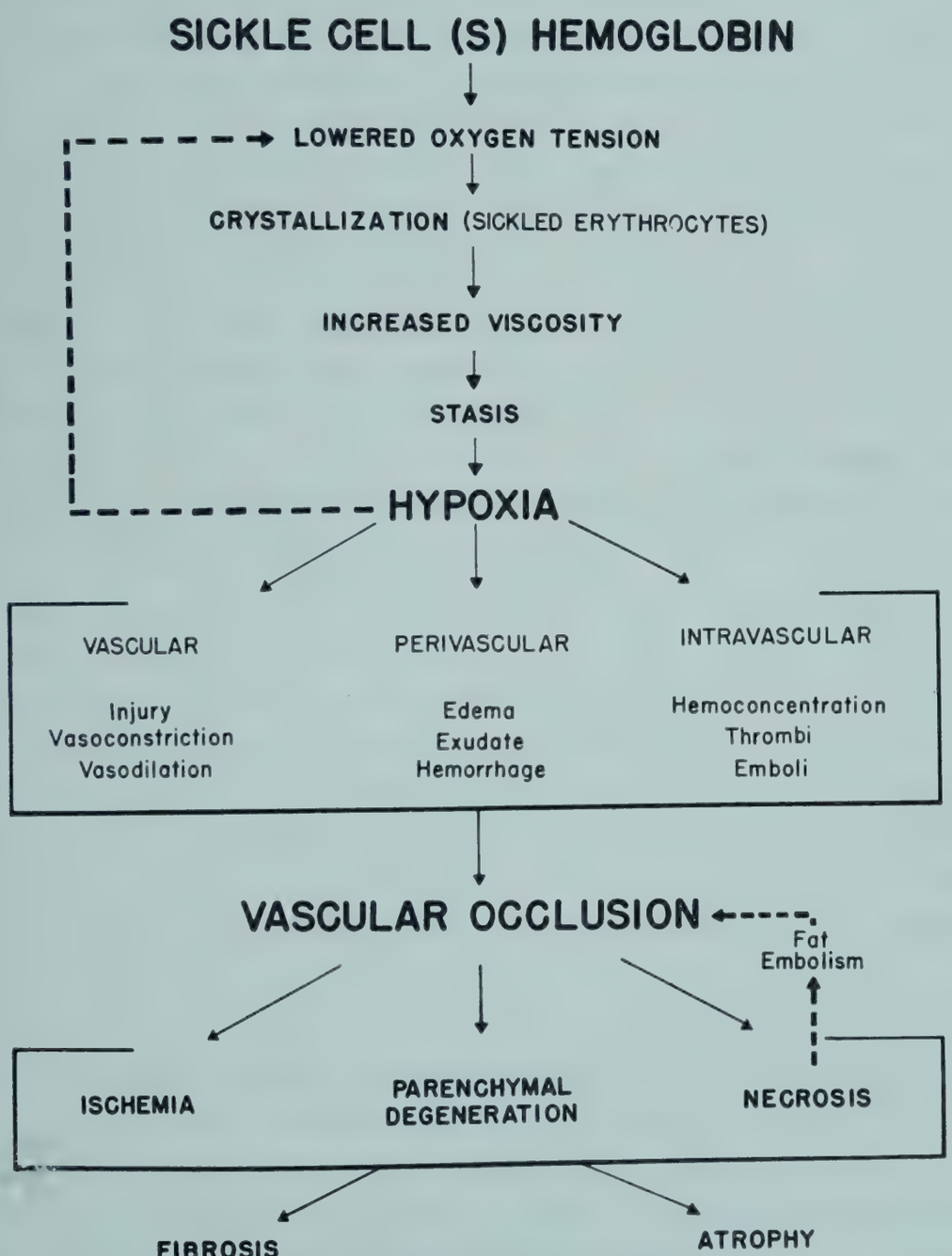


FIG. 3.7. Vascular occlusive mechanisms in sickle cell disease (J. Nat. M. A. 46: 46, Jan. 1954.)

considerable variation in the incidence in different African tribes and it may occur in 25 per cent or more of the population in certain areas. The abnormality is demonstrable in occasional Caucasians and in members of other races who have no known Negroid ancestry or demonstrable Negroid characteristics.

The majority of patients with the sickle cell trait (S-A) have no symptoms and physical signs that can be attributed to hemoglobin S. They have no anemia, no demonstrable changes in the air-exposed blood smears, no signs of abnormal red cell destruction and no abnormality in the leukocytes and thrombocytes. At autopsy the findings attributable to the sickle cell trait are the sickling of erythrocytes and evidence of stasis of blood in the spleen and other organs. Sickling probably occurs as a terminal event, for there is usually no evidence of abnormal pigmentation, thrombosis or parenchymal degenerative change.

Individuals with the sickle cell trait, on the other hand, are those "against whom the scales are loaded" for they are poor surgical and obstetrical risks and more susceptible than are normal individuals to infections and other diseases. In conditions characterized by severe hypoxia, systemic sickling and generalized vascular occlusion with pooling of the sickled cells in the greatly enlarged spleen and focal necrosis in the brain and other organs may occur, causing sudden and often unexpected death.

Sickle Cell Anemia (Homozygous Sickle Cell Disease, S-S Disease, Sicklemia, Sickle Cell Trait with Hemolytic Anemia). Sickle cell anemia is usually manifest in early childhood, produces protean symptoms and signs and usually causes death before the age of thirty. In addition to the chronic anemia and jaundice of the hemolytic type, there are recurrent febrile episodes associated with severe pains in any part of the body, but most frequently in the bones, joints and abdomen. Acute surgical conditions are often simulated and unnecessary operations performed.

The spleen undergoes progressive changes from congestive enlargement to siderofibrotic atrophy. Leg ulcers are common in adults and are resistant to treatment.

The bone marrow in sickle cell anemia contains relatively little fat. Red marrow is demonstrable in the long bones and in the small bones of the hands and feet. In early stages of the disease there is a tendency toward widening of the marrow cavity with a thinning of the cortex and osteoporosis, but as the disease progresses, sickling of the red cells causes vascular occlusion which leads to gradual fibrous tissue replacement of the bone marrow, focal areas of necrosis, abnormal calcification and dis-

tortion of the trabecular pattern. Loss of functioning marrow decreases compensatory regenerative capacity. Smears from cellular portions of bone marrow reveal an absolute increase in all cellular elements with the majority of nucleated cells at the more mature levels. There is a relative increase in nucleated red cells.

Blood smears of patients with sickle cell anemia are characterized by oval cells, elliptical cells, oat shaped cells, and target cells (Fig. 3.4). Typical and diagnostic sickled cells, which are long and narrow, sharply pointed at both ends, darkly stained, bent in the middle and with punched-out margins are, as a rule, difficult to find, although in some patients they may be numerous. Red cells vary in size and hemoglobin content. Diffusely basophilic cells are conspicuous and nucleated red cells and Howell-Jolly bodies are usually demonstrable. Stippled red cells may be present. There are occasional neutrophilic myelocytes and metamyelocytes and hyperlobulated neutrophils. There is an essentially normal ratio of neutrophils, lymphocytes and monocytes. Occasionally one sees monocytes with phagocytized red cells. Thrombocytes are significantly increased.

As a rule, one can make the diagnosis of sickle cell anemia by examination of the blood smear alone, but it is a good rule to insist on a special preparation for demonstration of the sickle cells before making the final diagnosis, for any type of severe anemia may be characterized by elongated and pointed cells which closely simulate sickle cells and individually cannot be distinguished. It is thought that the sickle cell, as seen in the air-exposed and stained blood film, is a cell which has undergone sickling in the body, has become irreparably elongated and cannot revert to normal shape when the blood is oxygenated.

The diagnosis of sickle cell anemia may be confirmed by electrophoretic studies (Figs. 3.8, 3.9). Homozygous hemoglobin S moves as a single component which is intermediate between hemoglobin A and hemoglobin C. Although there is no hemoglobin A in S-S disease, there usually is a varying amount of fetal (F) hemoglobin which in the proper buffer systems can be demonstrated (Fig. 3.8).

Other hematologic features of sickle anemia include a significant leukocytosis and thrombocytosis and an increase in reticulocyte count. The anemia is usually normocytic and normochromic with a tendency toward macrocytosis and hyperchromia. The laboratory findings characteristic of increased blood destruction and hemolytic jaundice are present. (See under "Excessive Red Cell Destruction".) The red cells are more resistant than normal to hypotonic salt solutions.

The survival time of erythrocytes in sickle cell anemia is significantly

reduced. In spite of a marked compensatory marrow regeneration there is chronic anemia. There is also a chronic jaundice of the hemolytic type. At the time of the so-called "crises" there is, as a rule, no precipitous fall in erythrocyte values nor evidence of increased red cell destruction as manifested by pigment excretion studies. The crisis is not, as has been frequently supposed, a "hemolytic crisis" but is a crisis due to obliterative vascular phenomena. The jaundice which is often increased at the time of the crisis is due to necrosis of the liver and impairment of liver function and is often of the obstructive or regurgitant type rather than hemolytic. Rarely one may have aplasia of the bone marrow due to virus infection or other cause which may result in a sudden

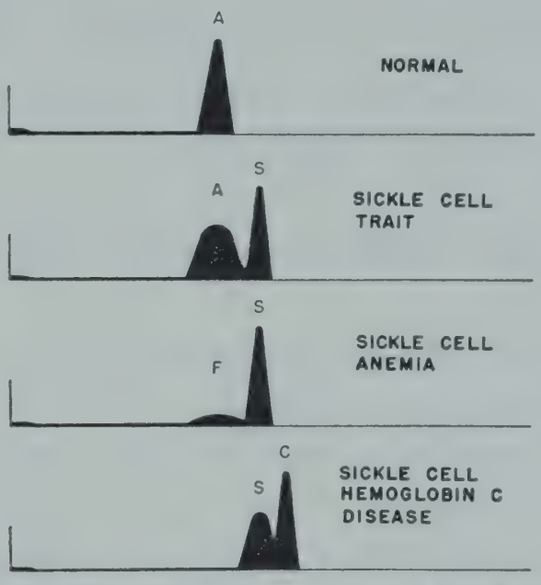


FIG. 3.8. Diagrammatic drawing showing standard electrophoresis patterns of different hemoglobins.

drop in erythrocyte values. The aplastic crisis is characterized by an absence of reticulocytes, a decline in the degree of jaundice and often leukopenia and increase in plasma cells.

No drug has proved to be of value in treatment of sickle cell anemia. Transfusions are of benefit during febrile crises. Splenectomy has been advocated for patients with large spleens, but proof of its efficacy has not been established.

Sickle Cell-Hemoglobin C Disease (S-C Disease). The sickle cell trait in combination with the hemoglobin C trait (Figs. 3.8, 3.9, 3.10) produces a disease which is usually less severe than classical sickle cell anemia (S-S). There is, as a rule, a mild type of hemolytic anemia with low grade jaundice, a slight increase in reticulocytes, occasional nucleated red cells in the peripheral blood and a hyperplastic bone marrow. Target cells are numerous in the stained blood smear but diagnostic sickle

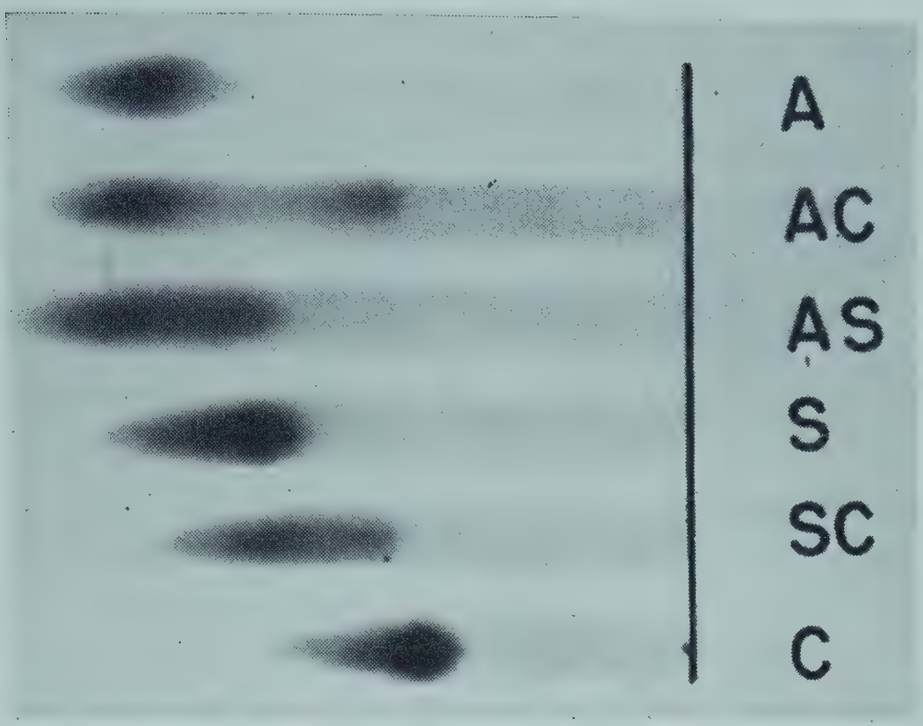


FIG. 3.9. Paper electrophoresis patterns of different hemoglobins

cells are few. As a rule, the spleen is moderately enlarged rather than atrophic.

Other Genetic Variants of Sickle Cell Disease (Fig. 3.10). The sickle cell trait may be combined with hemoglobin D and E or with other hereditary diseases such as thalassemia (sickle cell-thalassemia disease,

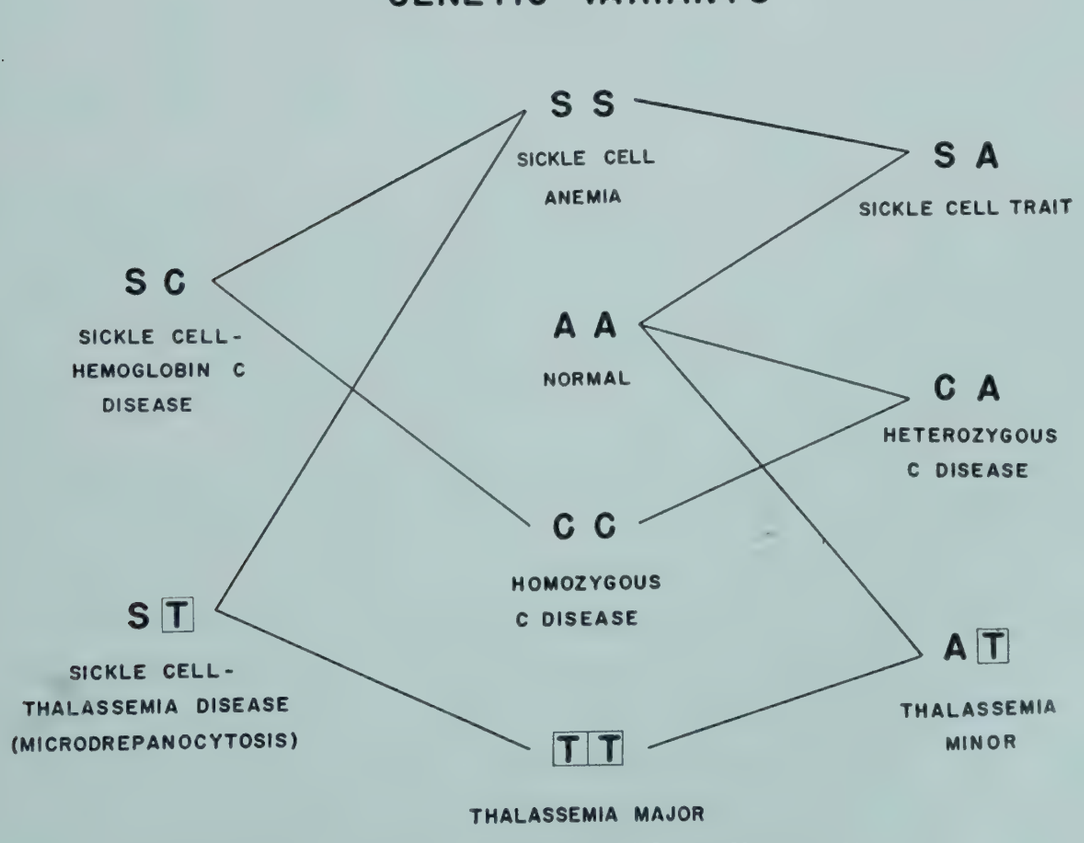


FIG. 3.10. Genetic variants of hereditary disease

microdrepanocytic disease), spherocytosis or ovalocytosis. The combination of hemoglobin S with these hereditary variants produces a disease which is, as a rule, less severe clinically and has a lesser degree of pathological change than sickle cell anemia but more severe than the sickle cell trait. The diagnosis of these conditions is based upon the combined clinical, hematological, electrophoretic and chemical studies and the examination of members of the family.

Hemoglobin C Disease. Hemoglobin C has an electrophoretic mobility and other chemical characteristics different from normal (A) hemoglobin and sickle cell (S) hemoglobin (Figs. 3.8, 3.9). The heterozygous form of hemoglobin C disease (C-A) occurs in approximately 2 per cent of Negroes. Homozygous hemoglobin C disease (C-C) is rare. The combination sickle cell hemoglobin and hemoglobin C (S-C disease) occurs in approximately 0.2 per cent of the Negro population in this country (Table 3.3).

Patients with heterozygous hemoglobin C abnormality (C-A) usually have no symptoms or signs attributable to their inherited abnormality except a variable number of target cells and increased resistance to hypotonic salt solutions. They are not anemic and have no signs of increased red cell destruction.

Patients with homozygous hemoglobin C abnormality have a mild anemia of the hemolytic type, low grade jaundice and signs of abnormal regenerative activity. The erythrocytes have a decreased survival time. Target cells are conspicuous in the stained smear. The spleen is enlarged. The hemoglobin in this disease crystalizes more readily than does normal hemoglobin.

Hemoglobin D, E, G and H. Recent intensive electrophoretic and chemical studies inspired by the research on S and C hemoglobin have revealed other abnormal hemoglobins such as D, E, G and H. The clinical and hematological changes produced by these hereditary variants have been inadequately studied.

Hereditary Leptocytosis. Hereditary leptocytosis is a gene-produced abnormality occurring most frequently among Mediterranean peoples in which there is a defect in hemoglobin formation and erythrocyte morphology characterized by thin cells or leptocytes which are more resistant than normal to hypotonic salt solutions. The flattened cells are irregular in shape and have folded and puckered surfaces. In some of the cells the elevations of surface occurring in the center of the cells appear as the bull's-eye of a target and are therefore called target cells (Fig. 3.4). Others have likened the central dark area which is surrounded by a

ghter zone and a peripheral ring to a hat and have called the cells Mexican hat cells."

If the condition is heterozygous there is no or slight anemia, but if omozygous, there is a severe anemia. Heterozygous carriers of the rait with mild anemia are diagnosed as **hereditary leptocytosis with mild ypochromic microcytic anemia** or **thalassemia minor.** In this condition he red cell count may be normal or greater than normal (erythrocytosis). The hemoglobin concentration is decreased. Other features include stippling of erythrocytes, a slight increase in reticulocyte count, occasional nucleated red blood cells in the peripheral blood and low grade aundice of a hemolytic type. The spleen may be palpable. The anemia, although hypochromic, does not respond to iron medication. The establishment of the diagnosis is important so that the patient will accept the condition as an inherited defect and will not be disappointed when anti-anemic drugs are ineffective and will not be put to the expense of repeated examinations for other diseases which may cause microcytic and hypochromic anemias.

Hereditary Leptocytosis with Hypochromic Microcytic Anemia (Cooley's Anemia, Mediterranean Anemia, Thalassemia Major, Target Cell Anemia and Erythroblastic Anemia of the Cooley Type). This disease usually becomes manifest in early infancy and causes severe disability. The bone marrow is hyperplastic and the marrow cavity is widened, producing thickening of the skull with striking "hair on end" trabeculations and osteoporosis of all bones, including the small bones of the hands and feet, with thin cortex and increased visibility of trabecular markings as seen in roentgenograms. The prominence of the facial bones and the yellowish type of pallor gives the so-called "Mongoloid" appearance. The spleen is enlarged. At autopsy there are the usual parenchymal changes associated with severe anemia and hemolytic jaundice. Iron-containing pigment in large quantities, resembling hemochromatosis, is demonstrable in many organs, and is in part due to multiple transfusions, which introduce iron in excess of bodily requirement.

The blood smear in patients with leptocytic anemia reveals grotesque shapes of erythrocytes, irregularities in hemoglobin within red cells and conspicuous target cells (Fig. 3.4). Anisocytosis is marked. Nucleated red cells in all stages of maturation, nuclear fragments, abnormal forms, and diffusely and punctate basophilic forms are conspicuous and give the picture referred to as "erythroblastotic." Leukocytes are increased.

Immature and hyperlobulated neutrophils are present. Thrombocytes are numerous.

Reticulocytes are increased and chemical tests for jaundice of a hemolytic type positive. In spite of the best medical care and multiple transfusions the anemia progresses. Death usually occurs during childhood.

Hereditary Spherocytosis (Familial Hemolytic Icterus, Congenital Acholuric Jaundice). In this disease there is an inherited erythrocytic anomaly, which is transmitted by either sex and which is characterized by spherical red cells which are destroyed more readily than normal in the circulation, producing jaundice of a hemolytic type. Depending on the compensatory regenerative ability of the bone marrow and the rate of red cell destruction there are varying grades of anemia. The symptoms and signs are manifest in early childhood and last throughout life. There are recurrent episodes characterized by rapidly developing anemia which in the past have been called "hemolytic crises" but which are due to a hypoplastic state of the bone marrow with failure in compensatory regeneration.

The spleen is enlarged and congested. Gall stones of the pigment type are common. There may be associated anomalies, such as tower skull. There is erythrocytic hyperplasia of the bone marrow with the majority of nucleated red cells at the metarubricyte level. The bones on x-ray may show osteoporotic tendencies of the type seen in thalassemia major. Leg ulcers may be associated. Other pathological changes are those common to hemolytic jaundice and chronic anemia.

The erythrocytes in the stained blood film appear smaller in diameter than normal but stain darkly because they are thicker (Fig. 3.4). There are many diffusely basophilic cells and occasional nucleated red cells. Variations in size and shape are present but not striking. There may be a few immature and atypical leukocytes. The reticulocyte count is characteristically increased, values often being above 10 per cent. Mean corpuscular values are within normal range or there may be a slight macrocytosis. Chemical findings on blood, urine and feces are those as described above for any hemolytic process. The Coombs test is usually negative. The spherical cells in this disease are less resistant than are normal cells to hypotonic salt solutions. The degree of fragility varies greatly in different patients and in the same patient at different times and is not related to the severity of the anemia, symptoms, or jaundice. Incubation of cells for a few hours at 37°C. may reveal abnormality which is not obvious at room temperature (Table 3.3).

Splenectomy is the treatment of choice and often leads to restoration

of normal balance in blood formation vs. destruction but it does not correct the spherocytosis or alter the osmotic fragility of the cells.

The disease, hereditary spherocytosis, is to be differentiated from familial non-hemolytic jaundice, which is a condition characterized by bilirubinemia apparently due to a higher threshold than normal for bilirubin excretion by the liver. Jaundice of variable degree without bilirubinuria and without increase of urobilinogen in the urine occurs. There is normal urobilinogen content of the feces and normal liver function. The spleen is not enlarged and blood smears reveal no abnormality.

Hereditary spherocytosis is to be differentiated from so-called "acquired hemolytic jaundice." This latter group includes conditions which have a varied etiology, prognosis and response to therapy. In both hereditary spherocytosis and acquired types of hemolytic jaundice there is evidence of anemia, increased red cell destruction, nucleated red cells in blood smears, and a high reticulocyte count. Spherocytosis and increased osmotic fragility are demonstrable in some but not all patients with acquired hemolytic jaundice. In some patients autoagglutinins and hemolysins may be demonstrated. The anemia, jaundice, splenomegaly and recurrent crises are usually manifest throughout life in the hereditary disease, whereas, onset of the acquired types is most likely to occur for the first time in adult life. In hereditary spherocytosis other members of the family are affected whereas in acquired hemolytic jaundice the family history is negative. In acquired hemolytic jaundice due to development of auto-antibodies the Coombs test is usually positive. Splenectomy is usually of benefit in the hereditary variety and is unpredictable in acquired types. Transfusions are said to be of spectacular value in treatment of certain acute hemolytic anemias, but in general there is likelihood of reactions and evidence of destruction of transfused cells in acquired types. The various conditions causing hemolysis of cells are listed under the appropriate etiologic heading.

Hereditary Ovalocytosis. In hereditary ovalocytosis the erythrocytes seen in stained blood smears or moist preparations are oval or elliptical (Fig. 3.4). In sealed moist preparations there is an exaggeration of the elongate shapes with blunt filaments but not sharp pointed projections of the type seen in sickle cell disease. The condition is inherited as a simple Mendelian dominant. The majority of persons with this trait are not in any way incapacitated, but approximately 10 to 15 per cent are said to have a mild anemia of a normocytic or slightly hypochromic

type with slight increase in the rate of red cell destruction and with well compensated blood formation.

Hemolytic Anemia of the Newborn (Erythroblastosis Fetalis, Icterus Gravis Neonatorum). This is a condition due to antibodies developed by the mother against Rh-Hr and other erythrocyte agglutinogens of the fetus and filtered back through the placenta into the fetal circulation (see Chapter 6).

Anemia Due to Unknown or Uncertain Cause

Hypoplastic Normocytic Anemia (Pancytopenia Due to Unknown Cause, Idiopathic Aplastic Anemia). Idiopathic aplastic anemia is a non-regenerative anemia due to unknown cause which occurs most frequently in younger individuals, does not respond to therapy and follows a progressively fatal course. The bone marrow is relatively acellular and the amount of fat increased. Smears of the bone marrow reveal few granulocytes, megakaryocytes, or erythrocyte progenitors. Nucleated cells that are found are mostly mature lymphocytes and occasional histiocytes, plasmocytes and undifferentiated mesenchymal cells. As a result of defective blood formation there is a peripheral pancytopenia. In addition to the symptoms of anemia there are hemorrhagic phenomena due to the thrombocytopenia and necrotizing lesions due to the lack of neutrophils. The majority of red cells are normal in size, shape and staining, and have a normal amount of hemoglobin. There may be occasional nucleated red cells in the peripheral blood due to a few remaining islands of erythropoietic tissue or to extramedullary foci. There is a relative lymphocytosis and a relative and absolute neutropenia with a shift to the left. The reticulocyte count is not increased.

Idiopathic aplastic anemia is to be differentiated from aleukemic leukemia, thrombocytopenic purpura and conditions which produce hypoplasia of bone marrow. In leukemia there is usually significant enlargement of the lymph nodes and spleen, blast cells may be found in the peripheral blood and the bone marrow is usually replaced by tumor cells. In thrombocytopenic purpura there is no anemia at the beginning of the illness and in later stages a regenerative anemia of a degree comparable to blood loss. Leukocytes in idiopathic thrombocytopenic purpura are increased with essentially a normal differential except for a neutrophilia with slight left shift. The bone marrow reveals increased cellularity with normal or increased numbers of megakaryocytes.

There are occasional patients who present the clinical and peripheral blood picture of aplastic anemia or pancytopenia whose bone marrow,

instead of being hypoplastic or aplastic, is normal or hyperplastic. Because the majority of such individuals do not respond to anti-anemic measures, the term "refractory anemia" is often applied to this group of diseases. Splenectomy and the use of Cortisone and ACTH have been advocated as treatment for this group of diseases and apparent benefit reported in some cases.

In some refractory anemias, there may be a prolonged and chronic course with the patient well adjusted to the anemic state and able to perform useful work in spite of a marked reduction in erythrocyte and hemoglobin values. Due to the growing size of the geriatric population and increasing exposure of all age groups to chemical and toxic agents, we may expect to find increasing numbers of non-regenerative anemias of undetermined cause.

Anemia, a Manifestation of Diseases Primarily Involving Systems Other than the Hemic System

Anemia is a manifestation of numerous diseases. As in diseases associated with infectious processes or due to chemicals, the blood picture is variable but the anemia is usually of the normocytic normochromic type with associated neutropenic and thrombocytopenic tendencies.

In chronic renal insufficiency anemia is a cardinal sign. The anemia is refractory to treatment. Anemia is common in rheumatoid arthritis, congestive splenomegaly, hypofunctional endocrine disorders such as myxedema, lupus erythematosus, in diseases of the gastrointestinal tract such as ulcerative colitis, and diseases of the liver and pancreas.

THE ERYTHROCYTOSES

General

Erythrocytosis is defined as an increased erythrocyte count of more than two standard deviations above the mean normal determined by the same method on the blood of healthy persons of the patient's age and sex group, and associated with an increased total blood volume.

Erythrocytosis Due to Known Cause

In thalassemia minor, in the early stages of chronic myeloid leukemia and in certain conditions in which there is exposure to cytotoxic drugs in small doses there may be a stimulated erythropoiesis with a slight augmentation of erythrocytes. Conditions which cause arterial hypoxia and erythrocytosis include high altitudes, congenital and acquired heart disease, and pulmonary disease such as emphysema, fibrosis or pulmo-

nary arteriolosclerosis (Ayerza's syndrome). In certain cases of chronic carbon monoxide poisoning or methemoglobinemia there may be a compensatory increase in red cells. The newborn child has a temporary erythrocytosis due to a low oxygen content of the circulating blood while *in utero*. After birth the stimulus for red cell production is removed and the red cell count rapidly falls.

An increase in the number of erythrocytes per cubic millimeter may occur as a result of the loss of plasma fluids such as occurs in shock or in conditions in which there is excessive sweating, vomiting or diarrhea with inadequate fluid intake (relative erythrocytosis). An increase in erythrocyte count due to anhydremia is not a true erythrocytosis since there is no increase in the total number of red cells.

One of the types of relative polycythemia known as stress polycythemia or Gaisboeck's syndrome is that seen in middle-aged individuals of the aggressive type, usually men, who are under increased stress and strain. They have a decrease in their total plasma volume and a relative, but not an absolute, increase in their red cell mass. The hematocrit in these individuals is within the limits of normal variation or only slightly increased. Individuals belonging to this group have pronounced masculine features as contrasted with the overfeminized individuals who often have apparent anemia due to an increase in the total plasma volume without an absolute decrease in red cells or hemoglobin.

Patients with stress polycythemia have symptoms closely resembling polycythemia vera. The symptoms include anxiety states, psychoses, dizziness, headaches, obesity, hypertension and chest pain. The spleen is not enlarged. There is increased likelihood of coronary thrombosis.

Erythrocytosis Due to Unknown Cause

Polycythemia Vera (Primary Erythrocytosis, Erythremia, Splenomegalic Polycythemia, Osler-Vaquez's Disease). Polycythemia vera is a chronic progressive disease of unknown cause, affecting adults and characterized by an overproduction of erythrocytes with an increase in total blood volume. The specific gravity and viscosity of the blood are increased, the velocity of blood flow retarded and thrombosis and infarction common. Patients afflicted with the disease have engorged blood vessels, a plethoric appearance, and splenomegaly. Because of circulatory incompetence, faulty aeration of the blood and occlusive vascular phenomena there are parenchymal degenerative changes.

The bone marrow is hyperplastic with extension of the red marrow into the long bones. The cell distribution is essentially normal. The

majority of the nucleated red cells are metarubricytes and the majority of neutrophils are segmented. Various authors have claimed that the bone marrow finding in polycythemia vera and erythrocytoses due to hypoxic states is significantly different, but criteria are ill-defined and non-specific. It has been the author's experience that the bone marrow is of little value in the diagnosis or differential diagnosis of polycythemia.

The blood in polycythemia vera is thick and dark and flows slowly through the venipuncture needle. Thin blood smears are difficult to make due to the increased cellularity and viscosity of the blood.

In the early stages of the disease the number of red cells per cubic millimeter, the packed cell volume, hemoglobin, total red cell mass and total blood volume are within the upper limits of normal variation, but as time goes on these values tend to increase. The diagnosis of polycythemia vera is established by exclusion of known causes, presence of splenomegaly, and clinical signs and symptoms plus the blood picture.

The stained blood smears from patients with polycythemia vera during the advanced stage of the disease reveal minimal poikilocytosis, anisocytosis, and occasional nucleated red blood cells. White cell distribution is essentially normal, but on searching one can usually find myelocytes and metamyelocytes. Thrombocytes are increased.

The mean corpuscular volume is decreased slightly and mean corpuscular hemoglobin and hemoglobin concentration are less than normal. The reticulocyte count tends to be normal or slightly increased. The sedimentation rate is slow. Serum bilirubin is usually moderately elevated and the urine urobilinogen test is positive. Clinical jaundice is seldom observed.

Spontaneous bleeding from mucous surfaces, ecchymoses and petechiae due to vascular distension, stasis, hemorrhagic infarction and vessel wall injury are common. Increased cell volume prevents normal clot retraction.

In the terminal phase of the disease there is a tendency for the mesenchymal cells to produce fewer red cells and more fibroblasts, with anemia replacing the polycythemia. Often there is extramedullary hematopoiesis with erythroblastotic and leukemoid peripheral blood pictures. In a small percentage of patients, myelocytic leukemia with the characteristic peripheral blood and tissue changes may develop.

One of the methods used to treat polycythemia is to remove blood periodically. The amount of blood to be removed is guided by hematocrit determinations. A good rule is to remove 500 ml. of blood as frequently as necessary to keep the hematocrit at approximately 50

ml./100 ml. Spray irradiation, radioactive phosphorus and cytotoxic drugs such as nitrogen mustard and acetylphenylhydrazine are also used.

HEMOGLOBINEMIAS AND HEMOGLOBINURIAS

Paroxysmal Cold Hemoglobinuria. In this condition there is intravascular hemolysis resulting in hemoglobinuria following exposure to cold. The phenomenon is caused by hemolysins which are activated by chilling and so affect the red cells that on subsequent warming, in the presence of complement, hemolysis occurs. The majority of patients with this condition have congenital or acquired syphilis and are benefited by antisyphilitic treatment.

Proof of the factor of cold in producing the hemolytic process is obtained by having the person immerse one hand or foot in ice water for 10 minutes and examining urine specimens before and after for albumin and hemoglobin (Rosenbach test). In patients with paroxysmal hemoglobinuria the urine darkens in 1 to 6 hours after chilling and the albumin and hemoglobin tests are positive.

Another procedure is to draw approximately 5 ml. of blood from the patient and another specimen from a normal person. The bloods are allowed to clot. Both tubes are placed in ice water for 10 minutes, followed by incubation in a water bath at 37.5°C. for 30 minutes. A positive test is characterized by a reddish discoloration of the supernatant serum of the patient and the absence of discoloration in the control tube. Tests for the demonstration of hemolysis following warmth after chilling are collectively known as Donath-Landsteiner tests.

Paroxysmal cold hemoglobinuria not due to syphilis may be caused by agglutination of the red cells in the cold, producing Raynaud-like syndromes with gangrene of the extremities, ears and nose. In this condition hemolysis occurs without subsequent warming, cold agglutinins are present in high titre and complement is not needed.

Paroxysmal Nocturnal Hemoglobinuria (Marchiafava-Micheli syndrome). In this rare disease there is a defect in the red cells which makes them more susceptible than normal to a slight lowering of the pH of the blood such as occurs during sleep. The degree of hemolysis is usually not severe enough to cause hemoglobinuria at all times but there is a chronic methemalbuminemia and hemosiderinuria and recurrent episodes of hemoglobinuria.

Hemolysis may be detected *in vitro* by incubating sterile defibrinated or clotted blood at 37.5°C. for six or more hours, during which time the pH has been lowered sufficiently to demonstrate hemolysis. Another method is to bubble carbon dioxide through defibrinated blood and to

incubate for 1 hour at 37.5°C. Control tubes of normal blood will reveal no or minimal hemolysis. Blood from patients with paroxysmal nocturnal hemoglobinuria will be hemolyzed under these conditions.

Exertional Hemoglobinuria (March Hemoglobinuria). In occasional individuals subjected to severe and prolonged physical exertion hemoglobinemia and hemoglobinuria may occur. Mechanisms involved are not known. Exertional hemoglobinuria causes minimal disability and anemia.

Hemoglobinurias Due to Miscellaneous Causes. Any condition characterized by jaundice of a hemolytic type may, if the blood destruction is rapid enough, result in hemoglobinemia and hemoglobinuria. Miscellaneous causes of hemoglobinuria are mismatched blood used in transfusion, water used in irrigation during transurethral prostatic resection, severe burns, certain bacterial infections, Oroya fever, yellow fever, malaria fever and particularly *P. falciparum* infections treated with quinine, sensitivity to the fava bean or pollen and toxins of certain snakes, insects and plant poisons.

Myohemoglobinurias. Myohemoglobin is a muscle protein related to hemoglobin which may be released in the blood stream and excreted by the kidney, in crush syndromes and in diseases in which there is muscle degeneration and necrosis.

FETAL HEMOGLOBIN

Fetal (F) hemoglobin is a peculiar kind of hemoglobin which is predominant during embryonic life and makes up approximately 50 to 90 per cent of the hemoglobin in the cord blood. The percentage of hemoglobin F gradually declines during the first months of life and usually by the end of the first year has been replaced by normal adult (A) hemoglobin. Although resembling hemoglobin A in many respects, hemoglobin F is chemically and immunologically different. It has a greater affinity for oxygen than does maternal adult hemoglobin and therefore favors placental transfer of oxygen.

When stroma-free hemoglobin solution is mixed with sodium hydroxide, the bright red oxyhemoglobin of the normal adult is changed to brown alkaline hematin within 1 minute, but when fetal hemoglobin is mixed with a strong alkali for 1 minute, it is resistant to this denaturation or change. The alkali denaturation test is the procedure of choice for the detection of hemoglobin F and for its quantitative measurement. Hemoglobin F can also be detected and measured by electrophoretic methods.

Fetal hemoglobin occurs in increased amounts in adults with various



PLATE 3.I

1. Spectrum
2. Hemoglobin
3. Oxyhemoglobin
4. Carboxy hemoglobin
5. Neutral methemoglobin
6. Alkaline methemoglobin



PLATE 3.II

7. Acid hematoporphyrin
8. Alkaline hematoporphyrin
9. Urobilin
10. Alkaline hematin
11. Acid hematin
12. Coproporphyrin I

Note: The absorption spectra are reproduced directly from observation with the use of the direct vision spectroscope. The yellow band has been slightly enlarged to facilitate reproduction of absorption bands occurring in this range. The scale in milli-micra has been adjusted to compensate for this enlargement.

types of hereditary anemias such as thalassemia major, spherocytic anemia and sickle cell anemia. It may be significantly increased in non-hereditary diseases characterized by increased hematopoiesis such as acquired hemolytic jaundice, untreated pernicious anemia and the myeloproliferative diseases.

ABNORMAL HEMOGLOBIN COMPOUNDS

Normally there are only two hemoglobin compounds, oxyhemoglobin in which the hemoglobin is oxygenated, but not oxidized, and reduced hemoglobin.

There are three abnormal conditions, carbon monoxide hemoglobinemia, methemoglobinemia and sulfhemoglobinemia, in which relatively stable hemoglobin compounds are formed which do not react reversibly with oxygen and therefore do not function as oxygen carriers. The effect produced is the same as that produced by anemia although there may be no reduction in red cell count or an actual increase due to compensatory regeneration of cells as a result of the hypoxia. The abnormal pigment is in the red cells and not in the serum.

Carbon Monoxide Hemoglobinemia. Carbon monoxide hemoglobin is formed when carbon monoxide is inhaled. Carbon monoxide combines with hemoglobin in the same proportion as oxygen to form a stable compound. The affinity of hemoglobin for CO is more than 200 times that for oxygen. Either acute or chronic carbon monoxide exposure is dangerous. When high concentrations of the gas are inhaled for a short time, death may quickly result from anoxia. Prolonged exposure to low concentrations may produce many symptoms referable to inadequate oxygenation of the tissues. Carbon monoxide poisoning produces an abnormally pink color of the skin since carbon monoxide hemoglobin is redder than oxyhemoglobin.

Carbon monoxide hemoglobin is slowly converted to oxyhemoglobin by the prolonged inhalation of a mixture of 10 per cent CO_2 and 90 per cent oxygen. Spectrophotometric or chemical analysis of the blood may be necessary to determine the cause of anoxia in carbon monoxide poisoning.

Methemoglobinemia. Methemoglobin is an oxidized hemoglobin having a chocolate brown color which may be formed when certain chemicals such as sulfonamides, phenacetin, acetanalid, bismuth subnitrate, nitrites or potassium chlorate are ingested or absorbed. Methemoglobinemia may occur as the result of wearing shoes recently treated with aniline dyes. Deaths have been reported in infants due to eating wax crayons, the use of unwashed diapers which have been stamped with

undry ink or as the result of drinking water from surface wells. In uch water the nitrogenous contaminants have been converted into ni-ites by soil bacteria. The previous boiling of such water increases the azard by concentrating the nitrites. Methemoglobinemia causes a ate gray skin color which is not associated with dyspnea and is not elieved by oxygen.

Sulfhemoglobinemia. Sulfhemoglobinemia is produced by a combina-ion of sulfides with hemoglobin. This combination may occur when xcessive amounts of an acetanalid or phenacetin sensitize hemoglobin o that it combines readily with H_2S. Sulfhemoglobinemia may cause narked cyanosis of a leaden blue type.

Sulfhemoglobinemia and methemoglobinemia may be detected spec-roscopically by diluting the blood with distilled water and observing he characteristic absorption in the red end of the spectrum (see Plates 3.I and 3.II). A drop of 10 per cent NaOH will cause immediate dis-appearance if the band is due to methemoglobin but will fade slowly if due to sulfhemoglobin.

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CHAPTER 4

LABORATORY TESTS USED IN THE DIAGNOSIS AND MANAGEMENT OF HEMORRHAGIC AND THROMBOEMBOLIC DISEASES

By L. W. Diggs

ESSENTIAL FACTS CONCERNING BLOOD COAGULATION

Observations During Coagulation

When blood escapes from blood vessels and comes in contact with air and with foreign objects, the soluble fibrinogen becomes converted into insoluble fibrin, and the physical properties of the blood change from that of a thick fluid to a firm, elastic, and fairly tough clot which retracts on standing. If the blood is examined microscopically during the process of coagulation, it is observed that some of the thrombocytes adhere to the glass and spread out along its surface while others stick to each other. The thrombocytes lose their well defined margins and become more granular. Multiple beaded, knobbed and branching cytoplasmic pseudopods and filaments extend from their surfaces. Long, needle-like fibrin structures appear. The needles coalesce forming strands. The thrombocytes that are not destroyed stick to the fibrin threads, tending to accumulate at the intersections. On standing the fibrin strands become bent and twisted.

When tissues are injured and blood vessels are cut or torn, the blood under vascular pressure escapes. The escape of blood lowers the hydrostatic pressure within the vessel and the seepage of blood into the tissues around the vessel tends to compress the walls. The injured vessels retract and their lumens become narrowed due to vasoconstriction. Clot-promoting fluids from injured cells, including blood cells and thrombocytes, mix with the outpouring blood. Thrombocytes, sticking to the ends of the injured vessels and along the wound tract, produce a mechanical blockade. Fibrin appears and forms interlacing and lattice-like strands that entrap the blood cells and block the outpouring blood. The clot on retraction tends to pull the edges of the wound and the blood vessel walls closer together and squeezes out serum which contains potent clot accelerator factors. The flow of blood ceases when the clot within the lumens of the ruptured vessels and the clot within the wound becomes attached and is of sufficient strength to exceed that of the vascular pressure. After the clot forms, it may be lysed or organized by the ingrowth of fibroblasts and new blood vessels.

Factors Involved

The principal factors in hemostasis and blood coagulation are:

1. Blood Vessel Factors
2. Tissue Factors
3. Thrombocytes
4. Plasma Factors
   a) Thromboplastin precursors (AHG, PTC and PTA)
   b) Prothrombin → thrombin
   c) Calcium
   d) Accelerator factors (Labile and stable factors and their precursors)
   e) Anticoagulants (inhibitors)
   f) Fibrinogen → fibrin
   g) Profibrinolysin → fibrinolysin

Theory of Blood Coagulation

The basic theory of blood coagulation which is almost universally accepted is:

$$\text{PROTHROMBIN} \xrightarrow[\text{calcium}]{\text{Thromboplastin}} \text{THROMBIN}$$

$$\text{FIBRINOGEN} \xrightarrow{\text{Thrombin}} \text{FIBRIN}$$

There is much difference of opinion about the part played by thrombocytes in the initiation and acceleration of the clotting process and about the details of activation and interreactions of thromboplastin factors, prothrombin conversion and accelerator factors. Research is handicapped by the difficulty in preparing pure substances, by the complexity of the process and by the dependence on the clotting time as the criterion of the concentration of the various factors. Innumerable terms for factors have been proposed without adequate proof that such factors really exist. Many different theories and "snakes by the tail" diagrams have been presented to explain the mechanisms involved and the interplay of various factors. The working diagram given in Fig. 4.1 represents the author's present concept of the more important relationships. This diagram does not include the vascular and tissue components in hemostasis nor the inhibitor and fibrinolytic systems.

Steps in the Coagulation of Blood

The sequence of events in the activation of the various plasma components and the formation of intermediate components are:

Mixture of blood with tissue fluids and exposure to air and to foreign surfaces
Agglutination and lysis of thrombocytes
Conversion of plasma thromboplastic precursors to thromboplastin
Conversion of prothrombin to thrombin
Conversion of fibrinogen to fibrin
Retraction of clot and expression of serum
} Activation of Accelerator Factors

The Autocatalytic Nature of the Coagulation Process

The coagulation of blood is characterized by chemical and enzymatic processes which rapidly gain momentum. Thrombocytes react with thromboplastin precursors to form thromboplastin and also aid in prothrombin and fibrinogen conversion. Thrombin on being formed apparently labilizes the thrombocyte factors and activates plasma factors which speed up the formation of new thrombin. Thus, there is a chain-like reaction or autocatalytic process which is characterized by chemical changes taking place at an ever increasing rate. These changes are not apparent for minutes but become explosively manifest when the viscosity of the liquid blood suddenly increases and immediately thereafter a solid clot is formed.

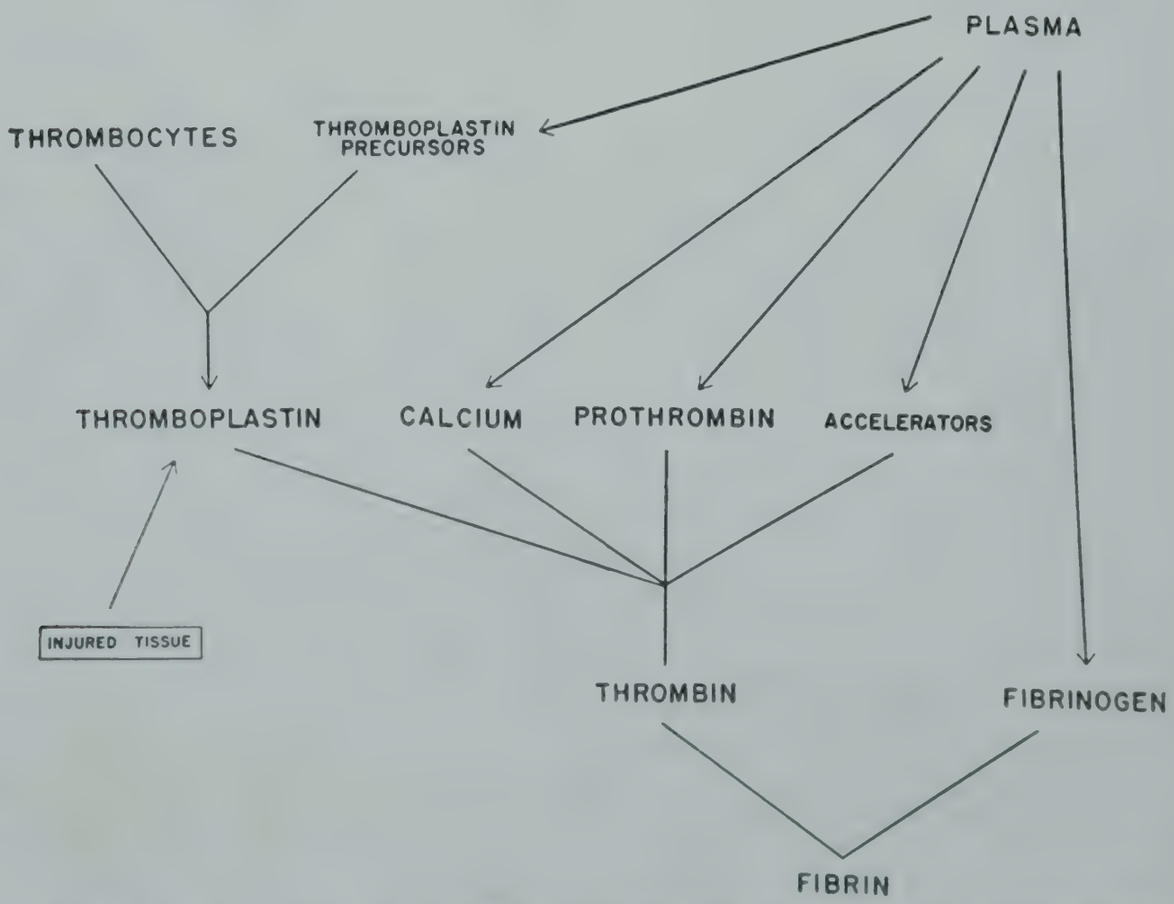


FIG. 4.1. Relation of principal factors involved in blood coagulation

TISSUE FACTORS

The tissues play an important role in hemostasis, for they contribute extracts containing thromboplastin which are essential in the initiation of the clotting activity. The perivascular connective tissue and collagen fibres and the fluids in the intercellular spaces create resistance to the outpouring of blood following breaks in the vascular wall. The elasticity of the skin and mucous membranes tends to automatically close wounds.

VASCULAR FACTORS—BLEEDING TIME—CAPILLARY FRAGILITY

The blood vessels play the predominant role in hemostasis, in preventing serious hemorrhage following trauma and in controlling spontaneous bleeding. This is accomplished mainly by the retraction of the vessels and by vasoconstriction. The stimulus for the vasomotor activity is supplied by the trauma and by the products liberated when tissues and cells, including thrombocytes, are injured. Vascular components in hemorrhagic disease are further discussed under "Hemorrhagic Diseases."

The tests which are most specific in detecting abnormalities in the smaller blood vessels are the skin bleeding time and the capillary fragility test.

THE SKIN BLEEDING TIME

General

The determination of the bleeding time is one of the most important of the procedures used in the detection and differential diagnosis of hemorrhagic diseases. It is the method of choice in the preoperative examination of patients scheduled for surgery.

Three procedures in common use include the estimation of the bleeding time from a puncture wound of (1) the finger tip, (2) the lobe of the ear (Duke's method) or (3) the skin of the forearm associated with venous stasis (Ivy method). The finger tip procedure is to be vigorously condemned for it may reveal no abnormality although there may be serious vascular disease. The skin of the finger is thick and elastic and closes over the wound made by the needle. The Duke method is more reliable than the finger puncture method for the skin of the ear lobe is thin and the subcutaneous tissue quite vascular. The ear lobe site is less convenient than the forearm, does not allow control tests and does not involve the stress of increased vascular pressure as does the Ivy method. The Ivy method is the procedure of choice.

The Ivy Method

Procedure: A blood pressure cuff is placed around the arm below the elbow and inflated to 40 mm. Hg. The skin of the pronator surface of forearm is cleaned with alcohol. In order to tense the skin at the proposed puncture site the skin is placed under tension by grasping the posterior portion of the arm between the thumb and finger. The skin is pierced to depth of 4 mm. with a "Hemolet" (American Hospital Supply Corporation). The drops are wiped away by means of filter paper at 30-second intervals until the bleeding has ceased. Filter paper makes an ideal absorbent material for this purpose (Fig. 4.2).

Normal: The free flow of blood varies from 1 to 6 minutes. Bleeding time between 6 and 10 minutes is indicative of a mild abnormality, and above 10 minutes is decidedly pathological.

Important Points

The "Hemolet" is an ideal instrument for this purpose for it has a guard in the proper position which insures a constant depth of puncture. (Spring lancets with guards should not be used unless they are sharpened and heat sterilized before each use.)

The drop of blood from the skin puncture wound should be at least 0.5 cm. and preferably 1 cm. in width. When the drop produced by the first puncture is less than 1 cm. in diameter, a second puncture adjacent to the first is recommended. The drops of blood from both wounds

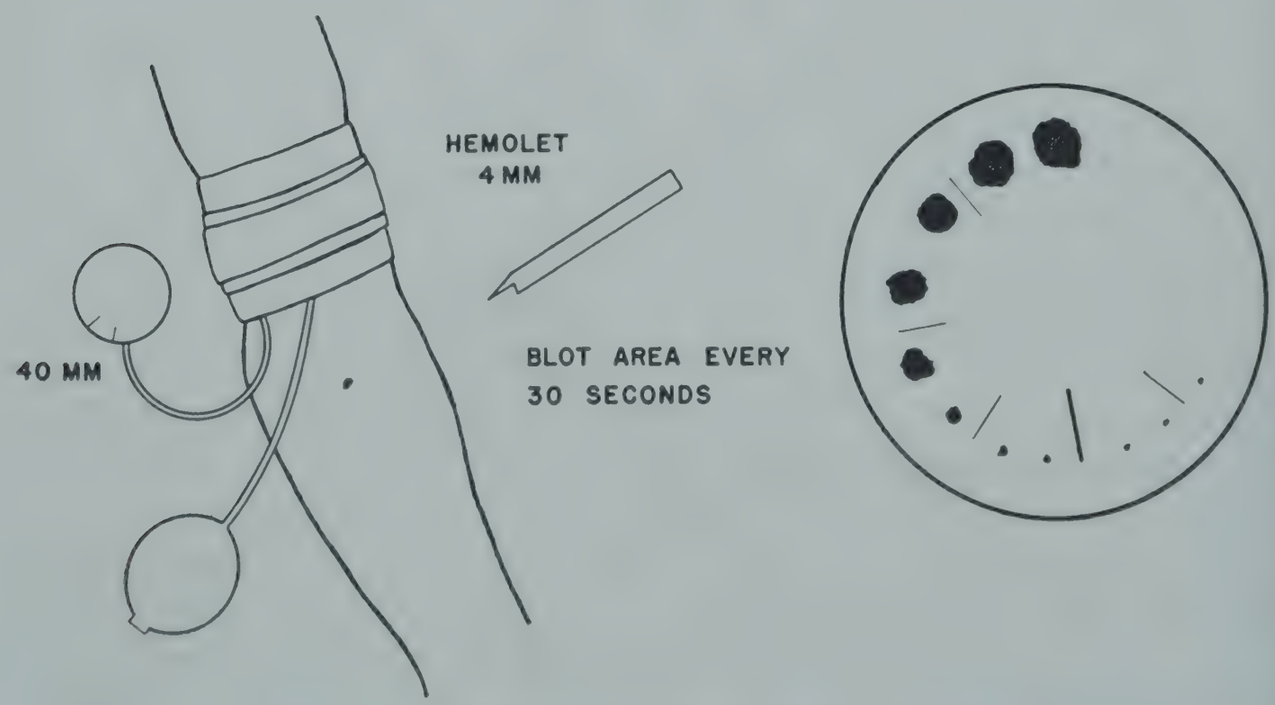


Fig. 4.2. Ivy bleeding time using "Hemolet"

are simultaneously absorbed on the filter paper. The average of the two is reported as the bleeding time.

The end point of the Ivy bleeding time is not always sharp for in many individuals there is an oozing of blood-tinged fluid from the wound for 1 to 5 minutes after the free flow of blood has ceased and the size of the drops has significantly decreased. Since normal individuals as well as patients with hemorrhagic disease may have prolonged oozing time, it is recommended that this phenomenon be ignored and that the end point be taken as that point at which the free bleeding ceases.

If the patient complains of pain in the arm during the test as the result of venous stasis or if the arm becomes cyanotic, the pressure in the cuff should be intermittently released. Indications for discontinuing venous stasis are the appearance of numerous petechiae and ecchymoses in the skin, the appearance of a hematoma under the skin at the puncture site or profuse bleeding when a vein or artery near the skin are pierced.

The test should be discontinued at the end of 15 minutes in those individuals who show no sign of cessation at this time. The continuation of the test after it has been determined that the bleeding time is significantly prolonged adds no additional information and has a bad psychic effect on the patient.

The errors are on the side of the test being normal rather than abnormal in the presence of disease. In case of doubt, where major surgery is contemplated, as in the operation for a congenital heart disease, one should perform a more severe test of trauma by actually making a skin incision and observing the flow of blood from a larger wound.

Sometimes there may be a recurrence of bleeding after a temporary cessation. This is always significant and should be reported.

Interpretation

The skin bleeding time is prolonged in systemic vascular diseases and in thrombocytopenic purpuras. The bleeding time is normal in AHG, PTC and PTA deficiencies, and is variable in non-thrombocytopenic purpuras, hypoprothrombinemia, and in deficiencies of stable and labile accelerator factors. A short bleeding time is indicative of normal hemostatic mechanisms.

The skin bleeding time must not be confused with the bleeding time from deeper tissues, from large blood vessels nor with the bleeding time from infected and necrotic surfaces for they are not necessarily correlated.

CAPILLARY FRAGILITY (TOURNIQUET) TEST

Capillary fragility (capillary resistance) procedures are tests of the ability of the small blood vessels to retain the red cells in their lumens under conditions of stress and trauma. Negative and positive pressure methods are used. In the negative pressure methods trauma is created by means of suction cups applied to the skin. Positive pressure methods are preferred. A tourniquet or inflated blood pressure cuff is placed above the elbow retarding the venous return and creating conditions of increased hydrostatic pressure and hypoxia within veins and capillaries. A rupture of one or more blood vessels constitutes a positive test.

There is no standardization of procedures nor well-defined normal values. All normal individuals will have a positive capillary fragility test if the positive pressure is maintained at a high level for a long time. Patients with vascular diseases, on the other hand, may have a negative test if the pressures are too low and maintained for short periods. The following procedures, with modifications based upon preliminary observations, are recommended.

1) If petechial spots have spontaneously appeared in the skin and/or mucous membranes, the capillaries are fragile and the capillary fragility is reported as positive without the performance of an additional stress test. No useful purpose is served by producing new and additional hemorrhages.

2) If, in the process of performing the Ivy bleeding time (see above) (40 mm. Hg, pressure for 5–15 minutes), petechiae appear in the skin below the cuff, the test is reported as positive and further tests are superfluous.

3) If no spontaneous petechiae are demonstrable and there are no petechiae after the completion of the Ivy bleeding time test, the blood pressure cuff is placed above the elbow of the arm opposite the one used for the bleeding time determination. The cuff is inflated to a point half way between the arterial systolic and diastolic pressures, with a maximal pressure of 100 mm. Hg. The pressure is maintained for 10 minutes. Petechiae appearing under the cuff, or in the skin in forearm or hand are reported as positive. It is not recommended that the number of petechiae in a given area be counted, for the test is too crude and subject to too many uncontrolled variables to make such an attempt at quantitaton valid or clinically useful.

Interpretation

The appearance of petechial hemorrhages beneath the skin following the application of a constriction band (Rumple-Leed sign) is a relatively

crude test for the ability of blood vessels to withstand minor trauma. In the test described above, certain normal individuals will show a few petechiae below the blood pressure cuff. Red-haired individuals are more likely to have positive tests and to bleed easier than blonds and brunets. Women have more delicate capillaries than men, and elderly people are more likely to have positive capillary fragility tests than younger individuals. The capillary fragility test is positive in thrombocytopenic states. The test may be positive in any of the conditions listed under vascular abnormality, systemic.

THROMBOCYTES AND COUNTING OF THROMBOCYTES

Function

The functions of thrombocytes or platelets in coagulation and hemostasis are:

1. To form aggregates which mechanically block the outflow of blood from injured blood vessels.
2. To supply
   (a) Thromboplastic factor (thromboplastinogenase) which reacts with thromboplastic precursors to form thromboplastin.
   (b) Vasopressor substance (serotonin).
   (c) Thromboplastin in small quantities.
   (d) Factor which aids in conversion of prothrombin to thrombin (Factor 1).
   (e) Factor which aids in conversion of fibrinogen to fibrin (Factor 2).
3. To aid in clot retraction.

Thrombocyte Counting Methods

There are no satisfactory or accurate methods for thrombocyte enumeration for thrombocytes are small, fragile objects which tend to stick to each other and to any wettable surface and are difficult to see and to distinguish from artefacts. In the hands of inexperienced technicians who perform occasional tests the counts are notoriously unreliable.

For clinical purposes and as a check on other thrombocyte counting methods, the examination of the ordinary stained blood smear and the enumeration of thrombocytes in 100 oil immersion fields yield reliable information. In normal individuals there are 300 to 1000 thrombocytes per 100 oil immersion fields. In thrombocytopenic states there are less than 100 per 100 fields, and often less than 10/100 o.i.f. On the other hand the finding of a few thrombocytes in practically every field and occasional clumps rules out thrombocytopenia at a glance and makes

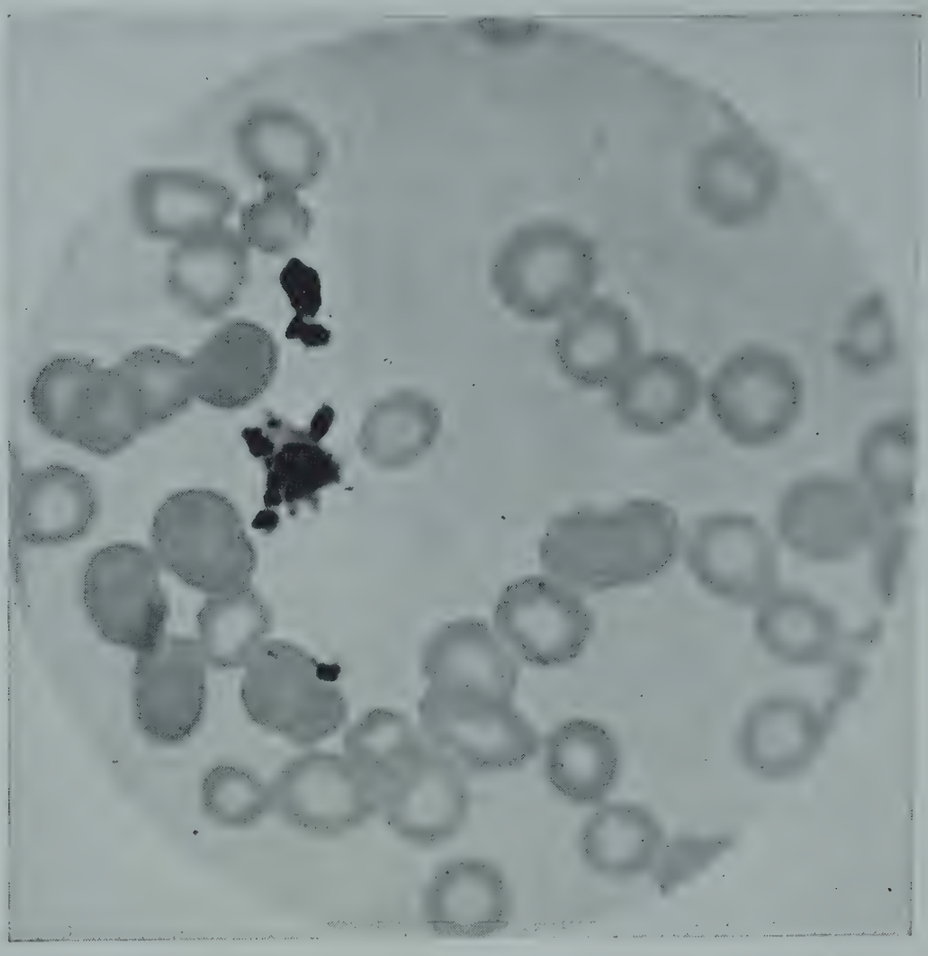


FIG. 4.3. Photomicrograph of thrombocytes in stained blood smear. Note the irregular shape and the tendency to form aggregates.

thrombocyte counting superfluous. In patients with aplastic anemia, leukemia or idiopathic or secondary thrombocytopenic purpura, who require frequent blood studies to determine prognosis and to guide therapy, the use of the simple blood smear for thrombocyte evaluation is more convenient and less expensive than direct counts (Fig. 4.3).

For the direct counting of thrombocytes the Rees-Ecker method is preferred (Chapter 1). The normal thrombocyte count by this method is 200,000 to 400,000 per cu. mm.

Interpretation

When the thrombocytes are decreased the bleeding time is prolonged, the tourniquet test is positive and the clot does not retract normally. The coagulation time in thrombocytopenic states, as determined by the capillary tube or single tube method is normal, but if the multiple tube method is used, the coagulation time is slightly prolonged. When silicone tubes or other more refined methods are employed there is a significant prolongation of the coagulation time in thrombocytopenic conditions. The prothrombin time by the one-stage method of Quick may be slightly prolonged due to a deficiency in thrombocyte factors 1 and

2. Prothrombin consumption in association with thrombocytopenia is low and the residual prothrombin in the serum increased. The tolerance to heparin is reduced when the thrombocytes are decreased.

The conditions characterized by thrombocytopenia are discussed under the heading "Vascular abnormalities, systemic, with decrease in thrombocyte count."

The thrombocyte count is increased following excitement, muscular exertion, adrenalin administration, ultraviolet light, hypoxia, hemorrhage and tissue injury. Following operation the thrombocytes are increased reaching a maximum in 6 to 10 days. In sickle cell anemia, in the convalescent stages of acute infections, in polycythemia vera and in the early stages of chronic myelocytic leukemia the thrombocyte count is characteristically increased. There are occasional patients who have persistent thrombocytosis due to unknown cause (idiopathic thrombocytosis). When thrombocytosis is marked there is a tendency toward intravascular thrombosis and infarction, but thrombosis is not predictable on the basis of the thrombocyte count alone. It is not unusual to have counts of 1,000,000/cu. mm. or more by the direct counting methods and 1,000 or more thrombocytes per 100 oil immersion fields without symptoms or signs of vascular occlusion.

PLASMA FACTORS—THE COAGULATION TIME

In addition to the tissue, vascular and thrombocytic factors in hemostasis, there are multiple plasma factors (thromboplastic precursors, prothrombin, calcium, accelerators, inhibitors, fibrinogen, and fibrinolytic components) which must be collectively and individually evaluated. The clotting time of whole blood or of recalcified oxalated plasma are screening tests for the composite action of all plasma factors acting simultaneously. The time required for the blood to clot is a function of the combined factors favoring coagulation on the one hand as opposed to the combined factors inhibiting coagulation on the other.

Methods for the determination of coagulation time may be divided into those which utilize blood from a skin puncture (micromethods) and those which utilize venous blood (macro- or test tube methods). Of the two the test tube methods are preferred.

Multiple Tube Method Using Venous Blood

Procedure:

One ml. of venous blood is placed in each of four dry serological tubes approximately 8 mm. inside diameter. The first tube is tilted at 30-second

intervals until the blood no longer flows down the side of the tube. Then the next tube is tilted at 30-second intervals until clotting occurs, following which the third and fourth tubes are in turn tilted. The time elapsed between venipuncture and clotting in the various tubes is noted. The average clotting time of the blood in the last three tubes expressed in minutes is reported as the clotting time.

The normal coagulation time with this method varies from 10 to 25 minutes. A clotting time of 25 minutes or more is indicative of a deficiency in blood coagulation components.

Capillary Tube Method

Procedure:

Freely flowing blood from a skin puncture wound is taken into several clean glass capillary tubes from 1 to 2 mm. in diameter. The tubes are held in the hand to keep them at approximately body temperature. At 30 second intervals the tube is broken. Ultimately there appears a fibrin strand that bridges the gap between the two separated parts. The interval of time between the collection of the blood and the appearance of the fibrin is the coagulation time (see Fig. 4.4).

The normal coagulation time with this method varies from 1 to 5 minutes.

Interpretation of Coagulation Time

Time required for the blood to clot after removal from the body is influenced by many factors. Mixture with tissue fluids increases the rapidity of coagulation; hence there is faster coagulation of blood removed by skin puncture than of blood removed by venipuncture and faster coagulation in venous blood removed with difficulty than in venous blood obtained by immediate entry of the needle into the vein and prompt withdrawal. Blood clots more rapidly in capillary and small



FIG. 4.4. The determination of coagulation time by capillary tube method. The capillary tube is broken between the fingers and the strand of fibrin is suspended between the broken edges.

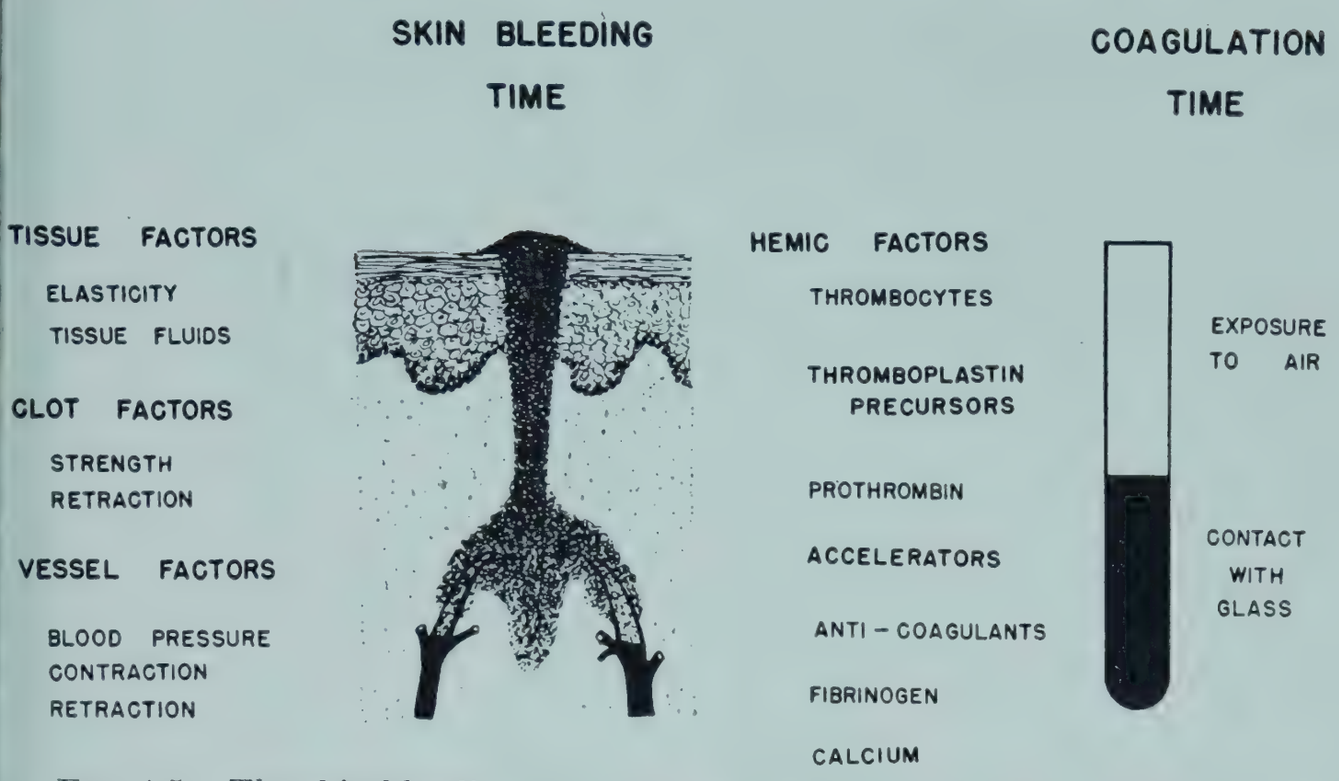


FIG. 4.5. The skin bleeding time and coagulation time, test tube method, are not comparable tests, for factors involved are not the same. The only factors the two tests have in common are the hemic factors.

tubes than in large containers. Blood clots slowly in the cold, and more rapidly at higher temperatures. Contact with air, dirt or with any wettable surface favors coagulation, whereas collection in absence of air, in oiled or in paraffin-coated containers, delays coagulation. Agitation of the blood favors fibrin formation, but stirring or whipping of the blood removes the fibrin and prevents clot formation. Oxalates, citrates, heparin and other substances prevent coagulation. The end points used in the various methods for determining the coagulation time are not the same, often are not sharp and are subject to errors in interpretation.

Because of these variable and often uncontrolled factors involved in blood coagulation, one must compare the results obtained with a given method with the range of normal variation for that method and must not interpret minor departures from normal as significant. Most of the sources of error in the methods used are on the side of shortening rather than prolonging the coagulation time and this is particularly true when micromethods are used.

The coagulation time is increased in any disease in which there is an absence of or small amount of any of the essential coagulation components (AHG, PTC, PTA, labile and stable factors, prothrombin or fibrinogen). The coagulation time is also prolonged in fibrinolytic diseases and in conditions in which there is an increase in inhibitor factors.

The coagulation time is poorly correlated with the thrombocyte count,

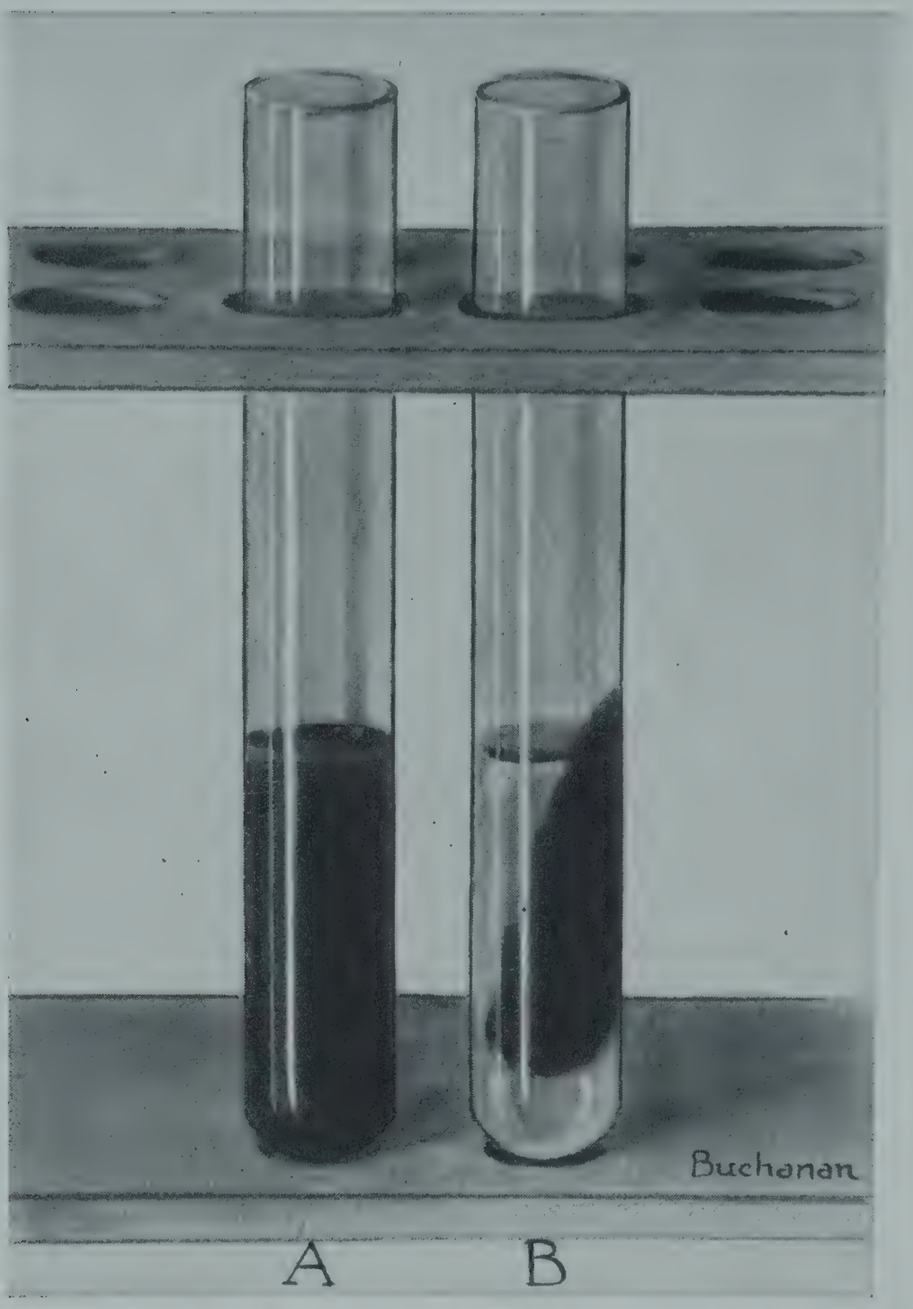


FIG. 4.6. A negative and positive clot retraction. The tube A shows no retraction of the clot. In tube B there is retraction as seen in normal blood.

bleeding time, capillary fragility test or the clot retraction. The apparent inconsistencies in the skin bleeding time and the glass test tube coagulation time are due to the fact that the two tests are not dependent on the same factors (Fig. 4.5). In the skin bleeding time the tissue and blood vessels play an important role in the stopping of the flow of blood, whereas in the coagulation time there is no blood vessel factor and the tissue factor is minimal. In the skin bleeding time the character of the clot is all important, for a feeble, poorly adherent, and non-retractile clot is incapable of closing the wound adequately and is swept away by the flowing blood as fast as it is formed, although it may be formed in the normal length of time. In the test tube coagulation time, one merely notes the time taken for the clot to form. The strength of the clot

and its ability to retract are not put to a test. The only factors that the bleeding time and clotting time have in common are the hemic factors (Fig. 4.5).

CLOT RETRACTION AND CHARACTER OF THE CLOT

The blood in the 4 tubes used in the estimation of the coagulation time is saved to observe the retractility and character of the clots. The actual timing of beginning clot retraction is not recommended as a standard procedure for the end point is not well defined, the results are not the same in various tubes, the observation requires too much time and little information of clinical value is obtained. As a rule retraction occurs within 30 to 60 minutes. In occasional normal bloods the clot may be so firmly attached to the sides of the test tube that the feeble retractive force cannot pull it away.

For special studies of the coagulation time and for research purposes the procedure proposed by Hirschboeck is recommended. In this procedure drops of whole blood from a skin puncture wound are taken up in a hemoglobin pipette and suspended in castor oil. The beginning of clot retraction is established "when a visible dimpling of the clot surface with extrusion of a tiny droplet of serum occurs". The average normal clot retraction time with this method is 33 minutes with a range of normal variation from 15 to 45 minutes. A retraction time of less than 15 minutes is considered indicative of a thrombotic tendency and a retraction time longer than 45 minutes indicative of hemorrhagic tendency.

Measurement of Serum Volume

Procedure:

Approximately 5 ml. of venous blood is placed in a graduated centrifuge tube or a 5 ml. Cutler tube. A wooden applicator stick, immersed in the blood before coagulation occurs, aids in removing the clot at the completion of the test. After coagulation has occurred the tube is placed in a water bath at 37°C. for one hour or left at room temperature for four hours. The total volume of the specimen is noted. The clot is carefully loosened, elevated, allowed to drain and then removed. The volume of serum left in the tube is measured. The serum squeezed out of the clot is reported in terms of per cent of original volume. The shape and consistency of the clot are noted (Fig. 4.6).

The error caused by the applicator stick and the error caused by the small column of red cells that settle to the bottom of the tube before

the clot forms, or cells that escape when the clot is lifted out, is usually less than 5 per cent of original volume and may be ignored.

Interpretation

A normal blood clot after maximal retraction is relatively dry, firm and maintains its moulded shape after removal from the container in which it was formed. A defective clot is one which is soft and soggy, is readily torn and after removal, flattens out as a shapeless mass from which the serum continues to drain.

The amount of fluid which is expressed from the clot is usually more than 40 per cent of the original volume. Values less than 40 per cent are indicative of a defective clot. The serum volume after retraction is decreased in thrombocytopenic states, in conditions in which there is defective thrombin formation and in conditions in which there is an increase in the volume of red cells as in polycythemia.

An escape of red cells from the clot as it is lifted from the test tube, causing the residual fluid left in the tube to be grossly bloody, is a sign of defective fibrin web which is incapable of holding the red cells tightly in its meshes. An estimation of the residual fluid in such a situation is unreliable as a measure of clot retraction.

Fluid Volume Per Cent of the Clot

The effectiveness of clot retraction in the presence of an anemia or erythrocytosis is evaluated by measuring the amount of fluid that is retained within the clot after maximal retraction (extracorpuscular clot volume). This is done by determining the packed cell volume of a portion of blood at the same time as the clotting time and clot retraction tests are performed. The serum remaining in the clot is calculated by subtracting the packed cell volume from the clot volume.

Example:

Volume of serum expressed from clot	40 ml./100 ml.
Volume of clot therefore is 100 minus 40	60 ml./100 ml.
Hematocrit (packed cell volume)	20 ml./100 ml.
Fluid volume per cent of clot = 60 minus 20	40 ml./100 ml.

The range of normal variation for the fluid volume per cent is 5 to 20 per cent with a mean of approximately 8 per cent. A fluid volume greater than 20 per cent is indicative of abnormality and hemorrhagic tendency. Clots which contain more than the normal amount of serum are flabby and weak, are easily dislodged and furnish poor supporting structure for fibroblastic organization.

COAGULATION TIME OF RECALCIFIED PLASMA

Blood is drawn from a vein and 4.5 ml. placed in a tube containing 0.5 ml. of 0.1 molar sodium oxalate (1.34 gm. of sodium oxalate in 100 ml. of distilled water). After thoroughly mixing, the tube is centrifuged at approximately 1,000 r.p.m. for 5 minutes. 0.1 ml. of supernatant plasma is placed in a test tube and 0.2 ml. of a 0.025 molar calcium chloride solution (2.77 gm. calcium chloride in 1,000 ml. of distilled water) is added. The tube is placed in a water bath at 37.5°C. and tilted at 15-second intervals, with minimal aggitation, until the plasma clots.

The normal coagulation time of recalcified oxalated plasma is 90 to 135 seconds.

The coagulation time of recalcified oxalated plasma is prolonged in conditions in which there is a prolongation of whole blood coagulation time. In severe thrombocytopenic states the time is prolonged.

PLASMA THROMBOPLASTIN PRECURSORS AND THROMBOPLASTIN

There are at least three thromboplastic precursors:

AHG (Antihemophilic globulin)
PTC (Plasma thromboplastic component)
PTA (Plasma thromboplastic antecedant)

A deficiency in any one of the thromboplastic precursors produces a hemophilia-like disease and is characterized by a delayed conversion of prothrombin to thrombin, defective prothrombin consumption, a prolonged coagulation time and abnormal hemorrhage following trauma The addition of thromboplastin in the one-stage method of Quick supplies the deficient factor and leads to a normal "prothrombin time." Fresh normal plasma containing all three precursor factors likewise corrects the deficiency in all and leads to a normal coagulation time in the presence of calcium.

AHG deteriorates rapidly after the blood is collected, whereas PTC and PTA remain active in serum and old bank blood. Barium sulfate absorbs PTC but does not absorb AHG nor PTA (Table 4.1). The differentiation of the three thromboplastin precursors is based upon the ability or failure of normal $BaSO_4$ treated plasma and normal 48-hour serum to correct the clotting defect in each case (Table 4.2). For details in performance of these tests the reader is referred to the article by P. G. Frick (J. Lab. & Clin. Med. 43: 860, 1954).

TABLE 4.1

Factors Present and Factors Absent or Deficient in 48-Hour-Old Serum and in Normal Barium Sulfate Absorbed Plasma

	Factors Present	Factors Absent or Deficient
Normal 48-hour serum	PTC PTA Stable factor	AHG Fibrinogen Prothrombin Labile factor
Normal $BaSO_4$-absorbed plasma	AHG PTA Fibrinogen Labile factor	PTC Prothrombin Stable factor

PROTHROMBIN

Prothrombin is a carbohydrate containing protein formed in the liver. Vitamin K is essential for its synthesis. Prothrombin circulates in the plasma as a pro-enzyme which is converted into thrombin. The concentration of prothrombin is estimated by various modifications of the so-called "two-stage" and "one-stage" methods. In the two-stage test the prothrombin is first converted to thrombin by the incubation of the plasma with thromboplastin and calcium. In the second stage the maximum thrombin yield is determined by measuring the time required for a standard fibrinogen solution to clot. The time is reported in terms of units per ml. Normal human plasma contains 250–400 units. The one-stage method of Quick is a more practical procedure and the one that is recommended for clinical work.

The One-Stage Prothrombin Method of Quick

Reagents:

1. Sodium oxalate solution (1.34 gm. sodium oxalate; 100 ml. distilled water)
2. Commercial thromboplastin-calcium mixture.* Follow directions of manufacturer in making up solution to be used in test.

Principle:

If it is assumed that fibrinogen and accelerator substances are normal in a given plasma and that inhibitor substances are not increased, and if

* Commercial thromboplastin reagents recommended include "Simplastin" (Chilcott), Solu-plastin (Schieffelin) "Thromboplastin" (Difco); "Permaplastin" (Hyland). For the preparation of thromboplastin from rabbit brain, it is recommended that the exact procedure described by Quick should be used.

TABLE 4.2

Effect of Normal $BaSO_4$ Plasma and Normal 48-Hour-Old Serum on the Clotting Defect in AHG, PTC and PTA Deficiency

Disease	Normal $BaSO_4$ Plasma	Normal 48-Hour Serum
AHG deficiency	Corrective	Ineffective
PTC deficiency	Ineffective	Corrective
PTA deficiency	Corrective	Corrective

thromboplastin and calcium are supplied in optimum amounts, prothrombin remains as the only variable in the system. The clotting time of the system, therefore, is a function of the prothrombin concentration.

It is obvious that the clotting time of a **plasma + thromboplastin + calcium system** is not a true measure of prothrombin when accelerator substances or fibrinogen and inhibitor substances are abnormal.

Procedure:

Obtain 4.5 ml. of venous blood and immediately mix with 0.5 ml. of sodium oxalate solution. Centrifuge. Using a standard 1-ml. micropipette, take 0.1 ml. of oxalated plasma from the centrifuge tube and place in a 13 × 100 mm test tube which is kept in a water bath at 37.5°C. The thromboplastin-calcium mixture is prepared by adding water to the dried powder. The suspension is placed in the water bath along with the tubes of plasma to be tested. Add 0.2 ml. of the warm thromboplastin-calcium suspension to 0.1 ml. of plasma and start the stop-watch. Rotate the tube in the water bath for 7–10 seconds. Then tilt the tube gently until the first fibrin web becomes visible. The time elapsed between the addition of the thromboplastin-calcium mixture and the appearance of the clot is noted. Care must be taken not to shake the tube too vigorously for the fibrin web may be broken up and escape detection.

Important Points

It is not necessary to withhold meals before the collection of blood for the prothrombin determination. It is essential to use the proper amount of blood and anticoagulant. Excess amounts of oxalate in relation to the blood collected results in a prolonged prothrombin time. Recently administered heparin or depot heparin will likewise cause an increase in the prothrombin time. If the tests cannot be performed immediately after collection, the plasma should be separated from the blood cells and stored in the refrigerator. The prothrombin time of plasma which is allowed to stand for more than 4 hours is significantly prolonged. It is necessary to use clean, dry syringes and needles, to perform a clean

venipuncture without admixture with tissue juices, to mix the blood thoroughly with the anticoagulant and to send the specimen immediately to the laboratory at a time when trained personnel are available. Glassware used in this procedure should not be used for other tests. Thorough washing with soap and water, rinsing with distilled water and drying is necessary. Detergents should not be used unless great care is taken in rinsing. Thromboplastic extracts should be refrigerated. Dilute thromboplastic solutions not used on a given day should be discarded or preserved by freezing. When commercial thromboplastin is used, the directions of the manufacturer should be meticulously followed. The use of a No. 22 nichrome wire loop which is moved across the bottom of the tube with a sweeping motion several times a second makes it easier to see the clot. An interval timer operated by a foot pedal facilitates accurate timing. Thromboplastic solutions that yield a clotting time of more than 15 seconds with normal plasma are unsatisfactory. At least two tests should be performed on each specimen. If the clotting times in the two determinations are not in close agreement, multiple tests should be made and the average time reported. It is necessary to perform control tests on at least one and preferably more than one normal plasma each day.

Dilution of plasma is thought by some to increase the accuracy of the test and to serve as a check on the standard procedure. Dilution techniques are not recommended, for they prolong and make more complicated an already difficult test, yield erratic results in pathological bloods and add confusion in interpretation.

Reporting. The clotting time obtained by the one-stage method of Quick should be reported in seconds and clotting time of the control normal plasma for that day also reported in seconds. The majority of workers recommend that the results also be reported in terms of so-called "prothrombin concentration per cent" which is obtained by the employment of a time-concentration curve. This curve is constructed by making serial dilutions of normal plasma with saline or prothrombin-free plasma (Fig. 4.7).

There is difference of opinion concerning the term "prothrombin concentration." The author is in agreement with those who feel that the percentage value obtained by means of the dilution curve should be called "clotting activity per cent" rather than "prothrombin concentration per cent," for the procedure reflects the combined action of all involved components and does not measure prothrombin alone. The fact that the percentage figure obtained is usually correlated with

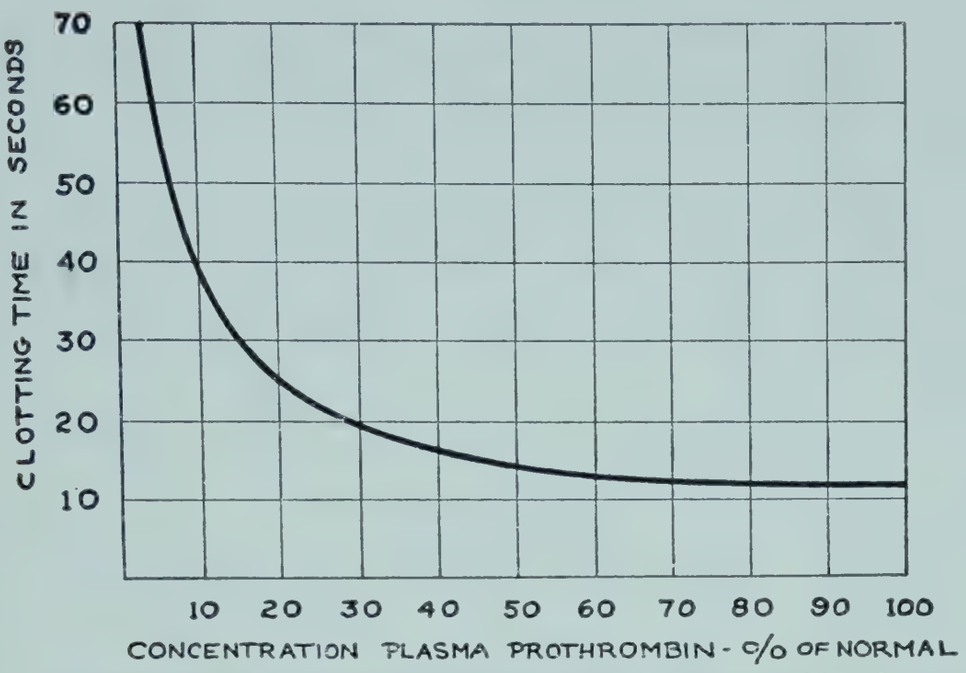


FIG. 4.7. An example of the time-concentration curve used in the estimation of the so-called "prothrombin concentration" in terms of per cent. (Curve constructed from figures furnished by Dr. A. J. Quick.)

the amount of prothrombin and yields figures which are clinically useful, does not justify a terminology that implies that it is an accurate or specific measure of prothrombin.

Other objections to the use of the time-concentration curve are:

1. The construction of dilution curves for each new batch of thromboplastin is technically difficult and expensive.

2. Variations of a few seconds in coagulation time are reported as marked differences in prothrombin concentration, which many physicians mistakenly interpret as significant.

3. The clotting activity curve obtained from plasma of normal individuals is not necessarily comparable to plasma from patients with hemorrhagic disease.

Normal Values. The coagulation time of normal plasma by the one-stage method of Quick varies with the thromboplastin used. With the most potent thromboplastic reagents the normal value is 11 to 13 seconds. Values between 13 and 18 seconds are acceptable for clinical use.

Bedside Method Using Blood from Skin Puncture

Procedure:

One drop of blood from a skin puncture wound is placed on a glass slide and rapidly mixed with an equal-sized drop of thromboplastin. The slide is held over a substage lamp to aid in visualization. A toothpick or a small glass rod is passed through the edge of the drop at 2–3 second intervals until fibrin is observed. Continual stirring is to be avoided for this may create

error due to defibrination. The duration of time from the drawing of the blood until the clot forms is noted by means of a stop watch. Controls should be run with blood of normal individuals. The clotting time of the patient and of the control should be reported.

Normal blood will clot in 15 to 25 seconds if potent thromboplastin is used. This procedure and other bedside methods are less accurate than the standard Quick procedure, but are recommended when there is difficulty in performing venipuncture or where the veins of the patient must be spared for essential intravenous therapy.

Interpretation

The clotting time of plasma to which thromboplastin has been added in the presence of optimum amounts of calcium is prolonged in diseases in which there is prothrombin deficiency (see "Hemorrhagic Diseases"). The one-stage test also reveals a prolonged coagulation time in diseases in which there is deficiency in accelerator factors, and fibrinogen, and in diseases characterized by fibrinolysis and an increase in anticoagulants including heparin.

In patients being treated with Dicumarol or related drugs, which interfere with prothrombin activity, the coagulation time by the one-stage method of Quick should be maintained 2 to $2\frac{1}{2}$ times that of the control. A clotting time 3 times that of normal plasma is indicative of hemorrhagic tendency. The plotting of the daily coagulation time on a graphic sheet enables the physician to follow at a glance the trend of events and to visualize the therapeutic response to the anticoagulant used. A form developed by Warner-Chilcott Laboratories (Morris Plains, New Jersey) for recording clotting activity in relation to therapy is an example of an acceptable graphic chart (Fig. 4.8). Patients receiving Dicumarol in the same dosage vary widely in prothrombin response. Therefore, it is necessary to check the plasma each day and to withhold medication until the result is known.

The one-stage method of Quick is of great value in differentiating the hemorrhagic diseases due to deficiencies in thromboplastin precursors from other diseases in which the coagulation time is prolonged (Table 4.3). In hemophilia and related thromboplastin precursor deficiency states the thromboplastin used in the test corrects for the deficiency and therefore gives a normal prothrombin clotting time.

SERUM PROTHROMBIN TIME

Principle. The phase of coagulation involving the reaction of thromboplastin and prothrombin is a stoichiometric process in which the in-

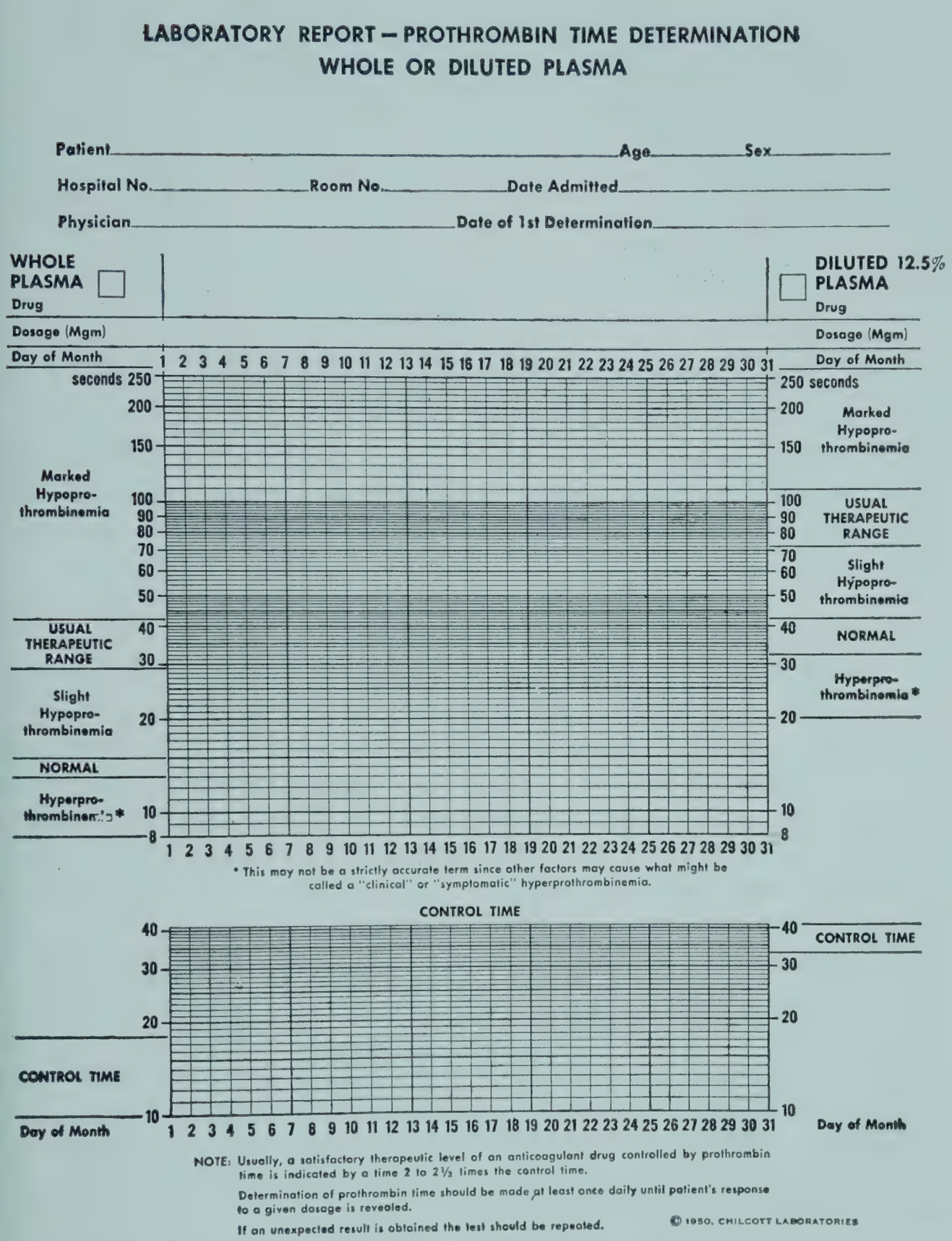


FIG. 4.8. A form of graphic chart used in plotting the prothrombin activity in relation to therapy. Courtesy of Warner-Chilcott Labs.

dividual factors are consumed. In the thrombin-fibrinogen reaction the thrombin acts as an enzyme or catalyst. The thrombin is not consumed in the process and a small amount may react over and over again, ultimately causing the conversion of all of the available fibrinogen to fibrin.

TABLE 4.3

The Differential Value of the One-Stage Method of Quick in Diseases Characterized by a Prolonged Coagulation Time of Whole *Blood*

Normal	Prolonged
AHG deficiency	Prothrombin deficiency
PTC deficiency	Stable factor deficiency
PTA deficiency	Labile factor deficiency
	Fibrinogen deficiency
	Excess anticoagulants
	Increased fibrinolytic activity

The amount of prothrombin that is converted into thrombin and the amount of residual prothrombin that remains in the serum after coagulation varies with the amount of thromboplastin and prothrombin that is available.

Reagents:

1. Thromboplastin plus calcium mixture (as for Quick one-stage method).
2. Prothrombin-free plasma. This reagent is prepared by adding 100 mg. $BaSO_4$ (Merck & Co.) to each ml. of fresh oxalated plasma (0.5 ml. 1.34 per cent sodium oxalate to 4.5 ml. blood). The mixture is shaken at room temperature for 5 minutes. This is centrifuged at 2,000 or more r.p.m. for ten minutes. The supernatant fluid contains fibrinogen and labile factor.

Procedure. Two ml. or more venous blood is collected in a clean and dry test tube (75 x 8 mm). Blood in tubes used for coagulation time is suitable. Clotted blood is centrifuged and supernatant is used. The serum is placed in water bath at 37°C. for 3 hours in order to inactivate the thrombin. Using pipets as in standard one-stage prothrombin test 0.1 ml. of barium sulfate treated plasma is mixed with 0.2 ml. of the thromboplastin-calcium suspension and 0.1 ml. of supernatant serum to be tested is added.

The interval between the addition of the serum and the appearance of the clot is the serum prothrombin time.

Normal Values. The normal serum prothrombin time is greater than 20 seconds, indicating that there is little prothrombin left in the serum. A serum prothrombin time less than 20 seconds indicates that there is an abnormal amount of unconsumed prothrombin left in the serum.

Reporting. The test should be reported in terms of seconds giving the control values for normal serum and for normal plasma.

If desired the prothrombin concentration in the normal plasma and in the serum can be estimated by use of a time-concentration curve and the prothrombin consumed during coagulation is calculated by the formula:

Prothrombin in plasma minus residual prothrombin in serum = prothrombin consumed

Interpretation

In the process of coagulation of normal blood 80 to 90 per cent of the prothrombin is consumed.

The prothrombin consumption is decreased in AHG, PTC and PTA deficiencies, in thrombocytopenic purpura and in some patients with thrombocytopathic purpura and with deficiencies in accelerator factors.

CALCIUM

Calcium is an essential constituent in normal blood coagulation, but the determination of the calcium content of the blood or the amount of diffusible or non-diffusible calcium has practically no value in the hemorrhagic diseases for in none of them is there a significant decrease in these substances. A decrease in serum calcium sufficient to interfere with blood coagulation is incompatible with life. Hypocalcemia of the severest grade, as in parathyroid tetany, is not associated with hemorrhage and hypercalcemia and thrombosis are not related phenomena. For these reasons the patient who is bleeding should be spared the expense of blood calcium determinations.

The administration of calcium in the treatment of hemorrhagic diseases is time honored and there is much clinical evidence indicating that it is of some value. The benefits that follow calcium therapy or the improvement in hemorrhagic symptoms that follow the temporary elevation of calcium when parathormone is administered are thought to be due to the effect of calcium in increasing the vascular tone and in lowering the permeability of the capillaries rather than to the effect of the calcium on the coagulating mechanism.

ACCELERATOR FACTORS

Accelerator factors are substances in the plasma or serum which hasten the rate of coagulation. Whether these substances exist in a pure form

TABLE 4.4

Synonyms for Plasma Coagulation Components and Diseases Associated with Deficiencies of Specific Substances

Factors		Diseases	
Preferred term	Synonyms	Preferred nomenclature	Synonyms
Thromboplastin Precursors			
AHG	Anti-hemophilic globulin	Hemophilia	Classical hemophilia
PTC	Plasma thromboplastic component	PTC deficiency disease	Christmas disease
PTA	Plasma thromboplastic antecedent	PTA deficiency disease	
Accelerator Factors			
Labile factor	Accelerin Factor VI Serum Ac globulin	Labile factor deficiency disease	Parahemophilia Owren's disease
Stable factor	Convertin Factor VII Spca (Serum prothrombin conversion accelerator)	Stable factor deficiency disease	Hypoproconvertinemia Spca deficiency disease

or as intermediary complexes of thromboplastin, prothrombin and other factors is not known. The accelerator factors occur in the plasma as relatively inert forms or pro-factors which become changed during the coagulation process into active forms. (Examples Factor V → VI, proconvertin → convertin.) In the discussion to follow the term used will be that of the active form, it being assumed that the term also includes the inactive precursor form (Table 4.4).

There are two accelerator factors, the labile and the stable factors. The activity of the labile factor decreases rapidly within a few hours after the blood is collected and practically disappears within 24 hours. The stable factor is essentially unaltered for 4 hours at 37°C., and remains active for weeks in serum or oxalated plasma stored in the refrigerator Table 4.1. The commonly used synonyms for the labile and stable factors and the diseases associated with specific deficiencies are given in Table 4.4.

Owren's concept of the accelerator activity is that thromboplastin reacts with the precursor form of the stable factor, in the presence of calcium, to produce the active form of the stable factor. The activated stable factor (convertin) together with calcium brings about a slow conversion of prothrombin to thrombin. Thrombin then activates the labile factor (accelerin) which further accelerates the reaction.

There is no direct method available for the measurement of prothrombin accelerator substance. Deficiency is suggested when the clotting time is prolonged, when the clotting time of plasma plus thromboplastin and calcium (Quick's one stage method) is prolonged, and when the addition of a small amount of normal blood restores the coagulation time to normal values.

A complete lack of accelerator factors causes a significant increase in the coagulation time of the blood, but there may be as in prothrombin deficiency a considerable decrease without affecting the whole blood coagulation time.

Deficiencies of accelerator factors are usually congenital or inherited. Concentrations of the stable factor are decreased in parenchymal liver disease and in patients treated with coumarin drugs. The administration of serum therefore would be rational in treating hemorrhages in these conditions.

ANTICOAGULANT OR INHIBITOR SUBSTANCES

Principle. In hemorrhagic diseases with a prolonged coagulation time the delay in clotting may be due to inhibitors of one or more of the coagulation components. The presence of such inhibitor or anti-coagulant substance is detected by mixing various quantities of the plasma to be tested with plasma from a normal individual, following which calcium is added. The recalcification technique is described under "Coagulation Time of Recalcified Plasma."

Procedure:

	Tube Number					
	1	2	3	4	5	6
	Plasma Volume in ml.					
Patient	0.2	0.4	0.1	0.1	0.1	0.0
Normal	0.0	0.1	0.1	0.2	0.4	0.2

After mixing the plasma to be tested in proportions as given above, 0.1 ml. is removed from each tube and placed in clean tubes in a water bath at 37.5°C., 0.2 ml. of calcium chloride solution added to each specimen and the clotting time noted.

Interpretation

In conditions in which the prolonged coagulation time is due to potent anticoagulants the addition of plasma in large proportions does not significantly reduce the prolonged coagulation time.

In conditions due to a deficiency of thromboplastin precursors, accelerator factors or fibrinogen, normal plasma added to the plasma from the patient to be tested would tend to correct the deficiency and produce a normal or shorter coagulation time of recalcified plasma.

FIBRINOGEN AND FIBRINOGEN CONCENTRATION

Fibrinogen is a labile plasma globulin, formed in the liver, which is converted into fibrin in the presence of thrombin. The gelification of the fibrinogen or clot formation is thought to be a molecular rearrangement of the linear fibrinogen molecules with end to end and cross linkages (tri-dimensionable polymerization). The concentration of fibrinogen in normal plasma varies from 200 to 600 mg./100 ml. with an average of 400 mg./100 ml. Phenomena due to fibrinogen deficiency are not manifest until the concentration is in the neighborhood of 75 mg. or less per 100 ml. A modified Campbell and Hanna method for the determination of fibrinogen is recommended. The fibrinogen in plasma is precipitated by sodium sulfite and the concentration determined by the biuret method.

A significantly decreased fibrinogen concentration is to be suspected whenever there is a prolongation of the coagulation time above 30 minutes associated with defective clot formation. In conditions characterized by severe bleeding as in hemorrhage associated with puerperal states or surgical procedures, significant fibrinogen deficiency may be quickly determined by the addition of thrombin to venous blood in a test tube. Test material for this is now commercially available.* Another test is the Sneider fibrin titer assay test (Am. J. Obst. & Gynec. 64: 141, 1952). Fibrinogenopenia can be ruled out if the clot is firm, retracts normally, and does not allow the escape of a large volume of red cells.

FIBRINOLYSIN

In addition to the coagulation components and inhibitor substances in the plasma, there exists in the blood an additional enzyme system which is concerned with dissolution of the clot after it has formed.

* "Fibrindex"—thrombin (human) 50 units, for critical fibrinogen determination. Ortho Corp., Raritan, N. J.

The precursor substance in the plasma is called profibrinolysin or plasminogen and the activated substance fibrinolysin or plasmin. A balanced system of activators and inhibitors is thought to be responsible for the rate of conversion of profibrinolysin to fibrinolysin.

Abnormal fibrinolytic abnormality is detected by observing the clot after it has formed. Dissolution of the clot within several hours is indicative of fibrinolytic activity. Fibrinolysis of normal blood does not occur for several days.

SELECTION OF PROCEDURES

Petechial spots, ecchymoses and spontaneous bleeding from mucous surfaces are usually manifestations of systemic diseases such as nephritis, tuberculosis or leukemia; hence the first diagnostic procedures should include the usual history, physical examination, X-ray and laboratory studies. Special inquiry should be made concerning hemorrhagic phenomena in other members of the family, unusual bleeding after tooth extraction, cuts and operations, ease of bruising, prolonged menstrual bleeding, hemorrhage from gastrointestinal or urinary tracts, allergic manifestations, exposure to chemicals, recent drugs taken and dietary deficiencies. Lymph node enlargement, splenomegaly, hemorrhages and necroses of mucous surfaces and eye ground changes should be noted. Careful study of blood smears for pathological cell types and bone marrow examinations may be of value.

The minimal hemorrhagic study should include the Ivy bleeding time, the capillary fragility test, the coagulation time by the multiple test tube method, the observation of the character of the clot and blood smear examinations.

A more complete hemorrhagic study should include the above named tests and in addition a thrombocyte count, quantitative measurement of the amount of serum expressed from the clot, prothrombin time and general hematologic study which includes a red cell count, hemoglobin estimation, sedimentation rate, packed cell volume, calculation of mean corpuscular values and reticulocyte count.

In rare instances it is necessary to test for inhibitor substances, fibrinogen and serum prothrombin and to perform differential tests for thromboplastic precursors and accelerator factors, but in the majority of cases the definitive diagnosis can be made with the more simple tests.

Routine preoperative hemorrhagic studies are not needed provided an adequate history is obtained, a physical examination performed and hematocrit or hemoglobin and smear examination made. The history

and physical examination are reliable. The so-called "routine hemorrhagic studies" which usually consist of a bleeding time from a skin puncture wound of the finger tip and a clotting time by a micro method from the same wound are not reliable. They may give the surgeon a false sense of security and fulfill the requirements of the hospital, but they do not protect the patient.

If there is a personal or family history of abnormal bleeding, or the patient thinks that he is "an easy bleeder," hemorrhagic studies are indicated. Tests for hemorrhagic disease are also indicated when there is spontaneous bleeding from any mucous surface, ecchymoses or petechiae. These studies should not be assigned to relatively untrained personnel, but should be either personally supervised by the physician or assigned to technicians with special training in this field who have ample time.

If routine preoperative hemorrhagic studies are required by the hospital or senior staff, the procedures recommended are the blood smear examination (including a description of red cells and thrombocytes as well as differential white cell count) and an Ivy bleeding time which automatically includes a mild test of capillary fragility.

HEMORRHAGIC DISEASES

The recommended terminology and coding of diseases characterized by hemorrhage is that given in the latest edition of "Standard Nomenclature of Disease." In the majority of instances hemorrhagic phenomena such as "purpura" or "epistaxis" or abnormal laboratory findings such as "hypoprothrombinemia" or "abnormality of bleeding time" are listed, not as diseases but as manifestations of a given primary disease.

For the purpose of appraising a given condition and as an aid in establishing a diagnosis, standard laboratory procedures are performed. From these findings it is usually possible to determine the nature of the abnormality.

Vascular Abnormality, Local

Physical trauma is the most common cause of local bleeding. As a rule the bleeding is more severe from arteries than from veins and more profuse when the vessels are cleanly cut than when they are torn or crushed. A small vessel in the nasal septum may repeatedly bleed because it is near the surface and repeatedly traumatized, because it cannot retract adequately and because there is little tissue thrombo-

plastin. A middle meningeal artery which is ruptured due to skull fracture may continue to bleed because it is fixed in bone and cannot retract, whereas a similar vessel ruptured in a muscle will give rise to a small hematoma.

Necrosis of tissue at the site of inflammatory reactions, such as occurs in tuberculous cavities, gastric ulcers, sinus tracts and at the sites of tonsillectomy or other operations, may lead to profuse capillary bleeding or to bleeding from arterioles which are caught in scar tissue and cannot retract. Post-operative bleeding may also be due to loosening of ligatures, dislodging of the clot or its dissolution by fibrinolysis.

Thrombosis of vessels or occlusion due to emboli or external pressure, may produce hemorrhagic infarcts or necrosis. Thus, apparent hemorrhagic phenomena may be due primarily to abnormal intravascular coagulation. Hemorrhage due to vascular occlusion must always be kept in mind when attempting to explain bleeding from lung, intestinal tract or genito-urinary system or localized petechiae or hemorrhages in the skin and mucous surfaces.

Defects in vascular walls as in aneurysm, varicose veins or arteriosclerosis often are the cause of hemorrhage. Increased vascular pressure causes an increase in the extent of the bleeding from any given break in the vascular wall. "Functional uterine bleeding" is due to endocrine imbalance which causes abnormalities in the endometrial blood vessels and in other uterine mucosal structures.

Tumors have defective vascular structures, defective neuromuscular mechanisms and are subject to infarction and necrosis, hence are often characterized by hemorrhage. Malignant tumors are more likely to bleed than benign, and tumors on exposed mucous surfaces which are subject to continual physical trauma and infection are more likely to bleed than more deeply seated lesions. Vascular tumors (angiomata) are particularly subject to bleeding when on exposed surfaces.

Various diseases of the skin such as multiple hemorrhagic sarcoma (Kaposi), purpura annularis telangiectodes (Majocchi) and progressive pigmentary dermatosis (Shamberg) are characterized by vascular abnormalities with localized hemorrhages and abnormal skin pigmentation. Local skin lesions may be an initial manifestation of systemic polyarteritis or other vascular diseases.

Hereditary Telangiectasia. In this disease there are localized areas in which the capillaries are dilated, usually without evidence of generalized capillary abnormality. As a rule there is a history of similar lesions in other members of the family. The angioma-like lesions are

often found on the lips, tongue, nasal septum or hands. They may appear anywhere in the body and are often the cause of epistaxis and unexplained hemorrhage from the respiratory, gastro-intestinal or genito urinary systems.

In all of these conditions due to local vascular disease the hemorrhagic studies reveal no abnormality. Negative results are not without value, however, for they tend to rule out generalized or systemic disease and direct attention to local pathology which requires local treatment.

Vascular Abnormality, Systemic, with Normal Thrombocyte Count (Non-thrombocytopenic Vascular Purpura)

Secondary Capillary Purpura (Vascular Purpura, Secondary). Hemorrhagic phenomena with a variable but usually prolonged bleeding time, a positive capillary fragility test and a normal thrombocyte count are observed in numerous conditions such as infections, exanthemata, poisonings and intoxications, systemic diseases and cachectic states. Neisseria meningitidis and Klebsiella pneumoniae septicemias are sometimes the cause of massive hemorrhagic phenomena. There may be associated adrenal hemorrhages, producing the syndrome known as "Waterhouse-Friderichsen Syndrome." Hypoxia due to any cause leads to endothelial injury and capillary hemorrhage. Fat and other types of emboli cause vascular occlusion, anoxia and multiple petechiae.

Capillary Purpura Due to Allergy (Allergic Non-Thrombocytopenic Purpura, Anaphylactoid Purpura). Purpuras may be due to sensitivity to various chemicals, foods and inhalants. On elimination of the offending agent the hemorrhagic phenomena disappear, to return again when the patient is exposed to the allergen. In many patients there is an associated history of urticaria, asthma, hay fever, skin lesions, arthralgias, recurrent headaches or abdominal complaints and a family history of allergy. Often there is an associated eosinophilia.

In the older literature two hemorrhagic syndromes, Henoch's and Schoenlein's purpura, were described which are now thought to be variants of allergic non-thrombocytopenic purpura due to sensitivity to streptococci and other organisms. These syndromes clinically resemble acute rheumatic fever, polyarteritis and acute hemorrhagic nephritis. In Henoch's purpura the bleeding is primarily into the gastrointestinal tract with associated abdominal cramps and symptoms and signs suggestive of acute abdominal conditions. In Schoenlein's purpura arthritis and skin ecchymoses are conspicuous features. The distribution of purpura in the latter condition is peculiar in that it tends

to be most marked on the pressure areas as the backs of the arms, shoulders and buttocks.

Fulminating types of purpura may occur, characterized by massive hemorrhages into the skin and viscera, gangrene of the extremities, shock and death. This syndrome, to which the name "purpura fulminans" is sometimes given, is probably a severe form of immuno-allergic disease in which the blood vessels are the target organs.

Hemorrhage Due to Vitamin C Deficiency (Scurvy). In the presence of vitamin C deficiency there is defective formation and maintenance of intercellular substances. As a result of this defect hemorrhages occur from the mucous surfaces. Sub-periosteal and deep muscle hematomas develop and sometimes there are hemorrhages under the scalp and in the skin. The cevitamic acid content of the blood and urine is decreased. Quantitative determinations may be made on such specimens but often are not necessary for diagnosis. If scurvy is suspected, the diagnosis frequently can be made from the history of inadequate diet and by the response of the bleeding phenomena to fruit juices and vitamin C concentrates.

Purpura Simplex. There are certain individuals, usually women of fair complexion with sensitive skin, who have structural weakness of blood vessels. These women bruise easily, often have isolated black and blue spots from minor trauma or from no known physical injury. They may have nose bleeds and minor bleeding from gums and menorrhagia. This condition is not serious and there is no tendency toward uncontrolled hemorrhage following operations.

Hereditary Capillary Purpura (Hereditary Hemorrhagic Purpura, Pseudohemophilia, von Willebrand's Disease). Hereditary capillary purpura occurs in both sexes, is transmitted by either parent and is characterized by abnormal bleeding and large ecchymoses following trauma. The bleeding time is prolonged, the tourniquet test variable. The coagulation time and other tests reveal no abnormality. Patients with this condition are poor operative risks, but death due to uncontrolled hemorrhage, hemarthroses and disability due to central nervous system and muscle hemorrhages are rare.

Senile Purpura. Elderly people often have discoloration of the backs of the hands and on top of the feet and shins due to minor trauma with rupture of small blood vessels. The tourniquet test may be positive, but other laboratory tests are negative. Loss of elasticity of the skin and deficiency in tensile strength of the perivascular supporting tissue are associated factors.

Vascular Abnormality, Systemic, with Decrease in Thrombocyte Count (Thrombocytopenic Purpura)

Secondary Thrombocytopenic Purpura. Thrombocytopenic purpura is usually secondary to and a manifestation of systemic diseases. Included in the list of diseases or conditions which may be characterized by a decrease in thrombocytes are severe infections and septic states, such as meningococcic and other types of septicemia, miliary tuberculosis and histoplasmosis, intoxications by cytotoxic chemicals and poisons of plant and animal origin, allergic sensitivity to foods, drugs and inhalants, irradiation and hyperthermia. Thrombocyte formation is defective in conditions in which the bone marrow is injured or replaced as in aplastic anemia, myelofibrosis, osteopetrosis, granuloma, lymphoma, leukemia, myeloma and metastatic malignancy. Enlargement of the spleen and thrombocytopenia are often related phenomena. Miscellaneous systemic diseases which may be characterized by a decrease in thrombocytes include cirrhosis of the liver, nephritis, lupus erythematosus, sarcoidosis, lipoid dystrophies, pernicious anemia and pituitary basophilism.

Petechial hemorrhages, spontaneous bleeding from mucous surfaces, prolonged bleeding time, positive tourniquet test, and defective clot retraction are features of all thrombocytopenic conditions.

Thrombotic Thrombocytopenic Purpura (Thrombocytopenic Acroangiothrombosis). Thrombotic thrombocytopenia is an acute febrile disease of unknown cause characterized by multiple platelet thrombi in arterioles, capillaries and venules with an associated thrombocytopenia. Clinically the disease is differentiated from idiopathic thrombocytopenic purpura by the fact that the vascular occlusive phenomena produce multiple and bizarre focal neurological signs and symptoms, pains in various parts of the body, splenomegaly and hepatomegaly, skin rashes, anemia, mild hemolytic type of jaundice, and leukemoid blood picture. It is assumed that in this disease the thrombocytes are formed normally but are decreased because they are agglutinated faster than they are formed. The bone marrow in this condition has been inadequately studied.

Hereditary Thrombocytopenic Purpura. In rare instances thrombocytopenia is an inherited abnormality. The symptoms and signs and laboratory findings are similar to those observed in other thrombocytopenic conditions.

Idiopathic Thrombocytopenic Purpura (ITP, Purpura Hemorrhagica, Essential or Primary Thrombopenic Purpura, Werlhof's Disease). This disease occurs most commonly in children but may have its onset in the

latter years of life. It affects both sexes. The number of attacks of bleeding and of purpura, the duration and severity of attacks and the prognosis are extremely variable. Some patients may have one attack and none thereafter, others a series of attacks and then cessation, others continual recurrences throughout life. Death may occur during the first attack or subsequent attacks. The typical onset is ushered in by hemorrhages into the skin or by spontaneous bleeding from the nose, gums or other mucous surfaces. Hemorrhage may occur in the brain, kidney, lung or other viscera, giving rise to localized symptoms. Headaches are indicative of cerebral hemorrhage.

Harrington and others have shown that some of the patients with idiopathic thrombocytopenic purpura have an active thrombocytopenic factor in their blood which agglutinates and destroys thrombocytes and perhaps injures the cytoplasm of megakaryocytes. This is thought to be a manifestation of an autoimmune mechanism. The occasional association of ITP with acquired hemolytic anemia and the beneficial effect of splenectomy and the administration of ACTH and cortisone are in line with this antigen-antibody concept.

The spleen is usually not palpable, and the presence of a large spleen is decidedly against the diagnosis of idiopathic thrombocytopenic purpura. The megakaryocytes of the bone marrow are present in normal or increased numbers but show evidence of cytoplasmic immaturity and defective thrombocyte formation (Fig. 4.9). After splenectomy the thrombocyte production tends to return to normal.

In idiopathic thrombocytopenic purpura the anemia is in proportion to blood loss. Following hemorrhage there is normal regeneration of erythrocytes. The white cell count is variable, but as a rule there is a slight neutrophilic leukocytosis. The decrease in thrombocytes is the most significant finding. During the hemorrhagic episodes the thrombocytes are usually less than 100,000 per cu. mm. and are often less than 10,000 per cu. mm. The bleeding time is prolonged and the capillary fragility test positive. The clotting time is normal or slightly prolonged. There is defective clot retraction.

In this disease, as in all hemorrhagic conditions, transfusion is the most effective way to control bleeding and to compensate for blood loss. Citrated blood is as effective as non-citrated whole blood. Fresh blood is more effective as a hemostatic agent than old bank blood. Blood collected in containers with non-wettable surfaces, as in plastic bags or siliconized bottles, is more effective in supplying well preserved thrombocytes than is blood collected in the standard glass containers.

ACTH and cortisone are often effective in causing a cessation of

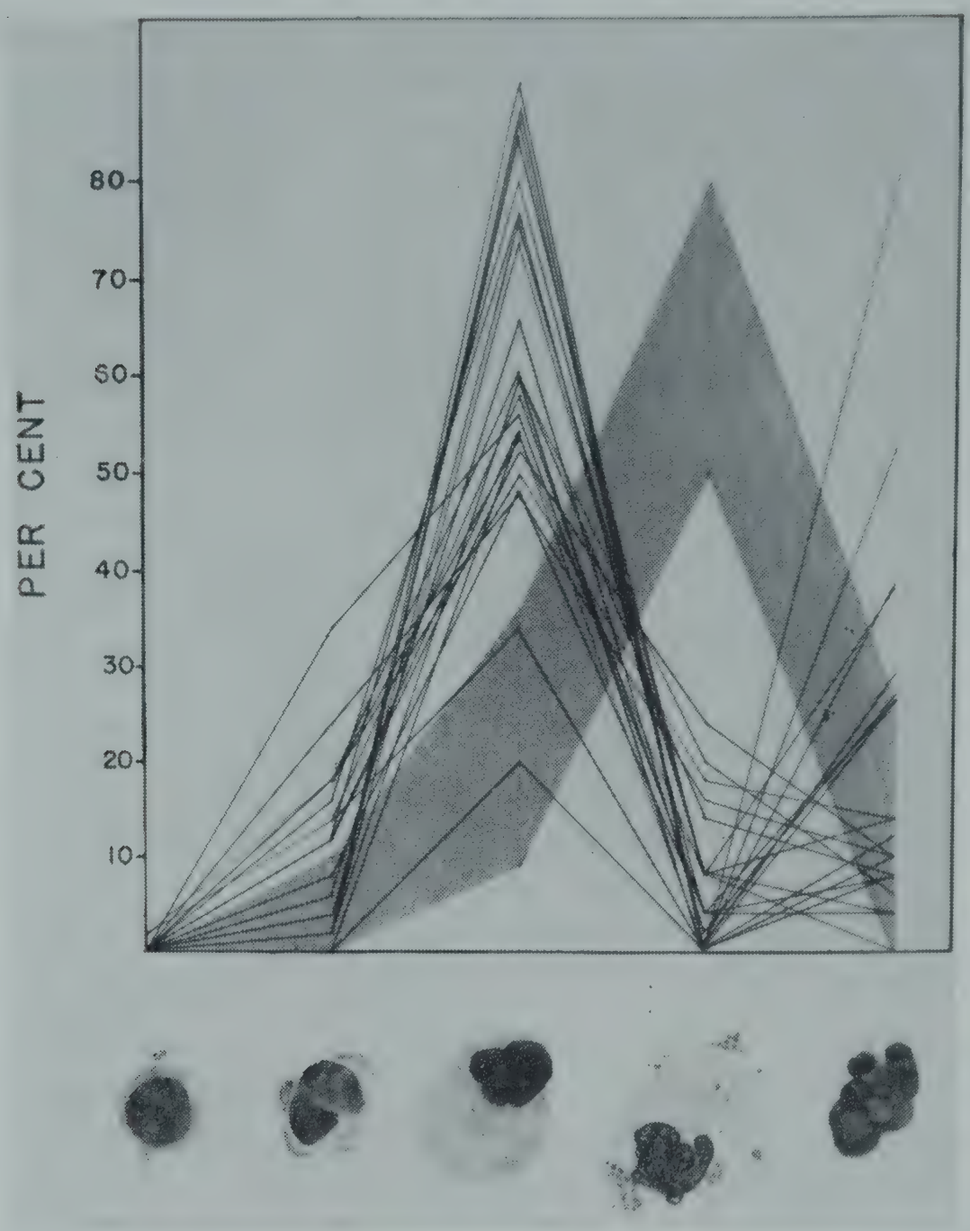


FIG. 4.9. Frequency distribution of megakaryoblasts, promegakaryocytes, megakaryocytes without thrombocyte production, megakaryocytes with thrombocyte production, and naked nuclei in sternal bone marrow smears of patients with idiopathic thrombocytopenic purpura. The shaded area represents the normal megakaryocyte distribution (Diggs and Hewlett, Blood 3: 109, 1948).

bleeding and a reduction of the bleeding time although it does not usually cause a dramatic change in the thrombocyte count.

In patients who do not respond to transfusions and steroid hormones and in adults with chronic thrombocytopenia and recurrent hemorrhagic phenomena, splenectomy is indicated. It is thought that the benfits that may follow the operation are due to the removal of an important site of thrombocyte destruction and antibody formation. Following splenectomy there is often a maturation of megakaryocytes with thrombocyte production which is mediated through a hypothetical circulating hormone. Contrary to statements frequently made in many text-books

splenectomy is not always followed by an increase in thrombocytes. Conservatism is recommended in children for the mortality with and without splenectomy is about the same. In adults with the chronic disease the mortality is significantly improved if the spleen is removed. There may be recurrences after initial improvement.

Tissue Factor Abnormality

Hyperelasticity of the Skin (Ehlers-Danlos Syndrome). In this condition there is an hereditary deficiency of elastic tissue which is manifested by hyperelasticity of the tendons and loose and hyperelastic skin. The slipping of the skin over the underlying structures leads to rupture of blood vessels and the appearance of localized hemorrhages which appear as discolored nodules. Hemorrhagic studies reveal no abnormality.

Deficiency in Plasma Thromboplastin Precursors

Hemophilia. Hemophilia is a relatively common hemorrhagic disease caused by a deficiency of antihemophilic globulin (AHG). The disease is transmitted as a sex-linked recessive character by female carriers who do not have manifest disease but who may have sons with the disease, normal sons, normal daughters or daughters who are carriers. The disease is practically limited to males for the possibility of the mating of a male hemophiliac and a female carrier is extremely remote. Sporadic cases are often observed in familes in which there is no known history of hemorrhagic phenomena.

Patients with hemophilia bleed profusely following minor trauma, have large ecchymoses and often hematuria or crippling hemarthroses. The hemorrhagic phenomena appear in early infancy and often result in death before adult years are reached.

The coagulation time of whole blood and of recalcified oxalated plasma is prolonged but varies in different patients and in the same patient at different times.

The clot that forms in the test tube in hemophilia has an abnormal appearance because the red cells settle out before the clot has completely formed. The clot contracts normally and expresses the normal amount of serum. Because of the deficiency in thromboplastin and the fact that the reaction of thromboplastin and prothrombin is stoichiometric, the prothrombin is not all utilized in the process of clotting. The supernatant fluid removed from a clot may continue to coagulate after the first clot is removed. Quantitative tests for prothrombin in the serum reveal poor prothrombin consumption.

When thromboplastin and calcium are added to plasma (Quick one stage method), the clotting time is normal, for the thromboplastin supplies that which is lacking and prothrombin, accelerator factors and fibrinogen are normal. The bleeding time in hemophilia is usually normal, for blood vessels retract and contract normally, the thrombocytes are normal and tissue thromboplastin is present. A recurrence of bleeding after temporary cessation may occur at the site of skin puncture and may be explained by the relaxation of the constricted blood vessels.

Fresh plasma given to patients with hemophilia supplies the deficiency and temporarily controls the bleeding but repeated injections may produce a refractory state thought to be due to anti-body formation. Thrombin applied locally to a bleeding point together with an elastic or other pressure bandage help in staunching flow when hemorrhage is from an accessible local site.

PTC Deficiency Disease (Christmas Disease). Until recent years hemophilia was generally considered as a single disease. Cross transfusions of patients with so-called "hemophilia" and in vitro mixing of the blood and plasma of patients revealed clinical benefit with a correction of the prolonged coagulation time in both parties. As a result of such investigations it is now known that hemophilia is not a single disease entity but a group of diseases characterized by a deficiency of at least three and perhaps more different types of thromboplastin precursors.

One of the variants of the hemophilia-like disease is called **Plasma Thromoboplastic Component (PTC) Deficiency Disease.** This type is less common than classical hemophilia and as a rule is less severe. Like hemophilia it is transmitted by a sex-linked gene and resembles hemophilia in its clinical and laboratory manifestations. Differentiation is based upon special tests (Table 4.2). Accurate diagnosis and differentiation is important for the hemorrhagic phenomena in PTC deficiency disease responds to old bank blood and serum as well as to fresh blood and plasma, whereas classical hemophilia requires fresh blood and plasma.

PTA Deficiency Disease. Rosenthal and others have described patients with hemophilia-like disease due to a plasma thromboplastin precursor which they have named *plasma thromboplastic antecedent.* This disease is not sex-linked. Differentiation from AHG and PTC is made on the basis of cross mixing experiments and differences in response to absorbed plasma (Table 4.2). PTA deficiency disease has been inadequately studied. It is the least frequent of the three thromboplastic component deficiency states and reported cases have been relatively mild.

Prothrombin Deficiency

Vitamin K Deficiency. Vitamin K, like vitamin D, is fat soluble and requires bile for its absorption. In chronic obstructive jaundice, bile is lacking in the intestinal tract, hence vitamin K is not absorbed and prothrombin in the blood is decreased. As a result hemorrhagic phenomena are common in patients with obstructive jaundice. The finding of a normal prothrombin time previous to operation does not give assurance that the patient will not bleed after operation. The anesthesia, surgical shock and anoxemia, the lessened intake of food, the blood loss and the loss of clotting components in the fibrinous exudates may lead to severe depletion of prothrombin after surgery.

In biliary fistula there is no jaundice, but there is inadequate absorption of vitamin K because of the lack of bile in the intestinal tract. The giving of vitamin K by mouth together with bile or the parenteral administration of vitamin K supplies the factor necessary for prothrombin formation.

In chronic diseases of the intestinal tract such as chronic bacillary dysentery, ulcerative colitis or in short-circuiting operations or intestinal fistula, there may likewise be inadequate vitamin K absorption.

Sulfa drugs and antibiotics by inhibiting the growth of intestinal bacteria may interfere with vitamin K formation.

Hypoprothrombinemia of the New Born. One of the most common of the hemorrhagic diseases in infants is hemorrhage of the newborn caused by *hypoprothrombinemia*. The prothrombin content of normal children at birth is lower than that of adults. The prothrombin concentration decreases progressively after birth for 3 to 6 days, after which there is a gradual return to the adult level. In some children the prothrombin is not reduced sufficiently to cause prolongation of the bleeding time or coagulation time, yet the patient may bleed severely following operation or minor trauma. In other infants the prothrombin may be greatly reduced and in these cases the bleeding time and coagulation time are prolonged, the clot is abnormal and there is spontaneous bleeding. The site of hemorrhage is usually the intestinal tract or umbilicus. Bleeding may occur in the skin, brain or other tissues. Hemorrhage due to prothrombin deficiency is rare at the time of birth and is usually maximal on the second to sixth day.

Multiple factors are involved in the hypoprothrombinemia of the newborn child. Inadequate prothrombin received from the mother, loss of prothrombin due to hemorrhage at the time of placental separation, defective formation of prothrombin due to the fact that the intestinal tract is sterile at birth and inadequacy of function of the immature

liver have been given as possible explanations of the prothrombin deficiency.

It is considered good practice to avoid tying the cord too soon thus allowing the child to receive all available placental blood, to allow the infant to nurse and to have fluids by mouth soon after birth. Transfusions and vitamin K are used in treatment.

Liver Disease, Severe. In severe liver disease as in chemical poisoning, diffuse carcinomatosis or the end stages of hepatitis or cirrhosis, the liver is unable to form prothrombin, even in the presence of adequate vitamin K. Often in liver disease there is in addition to prothrombin deficiency, a decrease in fibrinogen and thrombocytes and endothelial injury.

Drugs. Various coumarin drugs including Dicumarol, Tromexan and Wayfarin interfere with the formation of prothrombin and are used therapeutically in the treatment of thrombosing diseases. Individuals vary widely in their response to therapy, hence the necessity of performing the prothrombin time daily until the dosage has been stabilized.

Salicylates have an effect similar to Dicumarol in depressing the formation of prothrombin, but to a much less degree even in the presence of a maximal dosage. Hypoprothrombinemia following propylthiouracil has been reported.

Hereditary Hypoprothrombinemia. Rare cases of essential hypoprothrombinemia with hemorrhagic phenomena have been described.

Deficiency in Accelerator Factors

Labile Factor Deficiency Disease (Parahemophilia, Owren's Disease). Recurrent hemorrhagic phenomena and abnormal bleeding following trauma due to a deficiency of the labile accelerator factor is a rare congenital or familial disorder which affects both sexes. The coagulation time and "prothrombin time" are prolonged. The addition of small amounts of normal plasma corrects the coagulation defect. The thrombocytes are normal and the clot retracts normally. The bleeding time and prothrombin consumption are variable.

Stable Factor Deficiency Disease (Congenital Hypoproconvertinemia, Spca Deficiency Disease). Stable factor deficiency is associated with a clinical picture which clinically resembles hemophilia and labile factor deficiency disease. The differentiation between the various hemophilia-like diseases is based upon the prothrombin time and the effect on the coagulation defect when plasma or serum with known deficiencies of coagulation factors is added (Table 4.1).

Excess Anticoagulants

Numerous chemicals and certain poisons of plant and animal origin have anticoagulant properties in vitro or in vivo when administered to experimental animals, but these substances are seldom the cause of hemorrhages in man.

In occasional patients with atypical hemorrhagic disease the presence of excess anticoagulant can be demonstrated by the addition of the plasma to be tested to normal plasma. In the presence of coagulation inhibitor a prolongation of the coagulation time of the recalcified normal plasma is noted. Diseases characterized by excess anticoagulants are rare and have no specific name.

Excess Heparin. Heparin, according to Jorpes, is a mucoitin polysulfuric acid. It is thought to be formed in the tissue mast cells and delivered into the blood stream as one of the natural anticoagulant substances. Heparin is increased in certain patients exposed to ionizing radiation, in leukemia, in certain thrombocytopenic conditions and in anaphylactic shock.

On account of its acidic character, heparin combines readily with proteins of various kinds. The anticoagulant activity of heparin can be neutralized by the giving of protamin which is a basic protein.

Heparin by mouth has no influence on the coagulation time, but when given parenterally it causes a prolongation of the coagulation time and inhibits the agglutination of thrombocytes. It is generally agreed that heparin interferes with the thrombin mechanism, but is is disputed whether this drug acts as an antiprothrombin, an antithromboplastin or an antithrombin.

When heparin is given intravenously the effect is prompt and of short duration, hence the necessity of continuous administration. The speed of administration is guided by determinations of coagulation time at 4 hour intervals. Sufficient heparin should be given to maintain coagulation time that is two to three times the normal value for the method used. Heparin is often used in combination with Dicumarol. The heparin is given for the first 1 to 2 days until the Dicumarol effect is demonstrable, then the heparin is discontinued.

Fibrinogen Deficiency

Hereditary or Congenital Hypo- and Afibrinogenemia (Hereditary and Congenital Fibrinogenopenia). In rare instances patients are seen who inherit or are born with a marked decrease in fibrinogen or a complete absence of this essential clotting component. They have normal

thrombocytes and normal vascular mechanisms and may survive for years in spite of the fact that their blood is incoagulable or has an extremely long coagulation time with defective clot formation. They have recurrent episodes of severe hemorrhage following trauma and are temporarily benefitted by fibrinogen replacement therapy.

Fibrinogenopenia Associated with Obstetrical Conditions. In conditions such as placental separation, abortion, retroplacental hematoma, dead fetus and toxemia there is infusion of products of placental and fetal origin into the maternal circulation as the result of uterine contractions. These substances cause intravascular coagulation and disseminated fibrin emboli. The available fibrinogen in the plasma is converted into fibrin faster than it is formed, resulting in severe fibrinogen deficiency. In fetal death due to Rh and ABO incompatibilities the uptake of large amounts of antigen suddenly into the circulation of a sensitized mother may be an additional factor in producing shock-like states.

Prompt recognition of the condition, the giving of fibrinogen or large amounts of plasma and the emptying of the uterus are imperative, for the patients bleed profusely from every site of vascular trauma and rapidly pass into a state of irreversible shock.

Fibrinogenopenia Associated with Surgery. At the time of and shortly following major operations, the patient who has previously had no evidence of hemorrhagic abnormality may begin to bleed profusely. This condition is associated with systemic intravascular coagulation (defibrination) caused by tissue thromboplastic substances. The phenomenon is most frequently encountered in extensive operations on the lung and pancreas and prostate. Fibrinogen intravenously is indicated.

Fibrinogenopenia Associated with Miscellaneous Diseases. Included among the conditions which may be characterized by prolonged coagulation time, fibrinogen deficiency, and defective clot with varied hemorrhagic phenomena are shock states, plasma and whole blood transfusion reactions, severe burns, septicemias, leukemia, metastatic malignancies, liver diseases and severe nutritional deficiencies. In these conditions the fibrinogenopenia is usually terminal and is one of multiple abnormalities, including thrombocytopenia, increased capillary fragility, increase in anticoagulant substances and activation of fibrinolysins.

Abnormal Fibrinolytic Activity

In shock states fibrinolytic activity is increased, resulting in destruction of fibrinogen and incoagulable blood or rapid lysis of the fibrin in the clot soon after it has formed.

Fibrinolytic factors are demonstrable in cirrhosis of the liver and other parenchymal liver diseases and in prostatic and pancreatic carcinoma. ACTH and cortisone apparently are of benefit in temporarily decreasing the fibrinolytic activity.

Diseases Due to a Combination of Factors

Many hemorrhagic states are due, not to one factor alone but to a combination of factors. Thus in parenchymal liver disease we may have prothrombin and fibrinogen deficiency, labile and stable factor deficiency, increased capillary fragility, excess in anticoagulants and increased amounts of fibrinolysin. Coumarin drugs, while primarily affecting the prothrombin, also may be associated with alterations in vascular mechanisms and stable factor deficiency. In shock states there are vascular lesions, a decrease in thrombocytes and in fibrinogen and an activation of fibrinolysin.

Diseases Unclassified

Thrombocytopathic Purpura (Hereditary Hemorrhagic Thrombasthenia, Glanzmann, Thrombasthenic Purpura). In thrombocytopathic purpura the thrombocytes are large and atypical and defective in essential functions although present in normal numbers. The bleeding time is variable but usually prolonged which is in part due to deficiency in vasoconstrictor factor (serotonin). The plasma prothrombin time may be slightly prolonged and the prothrombin consumption less than normal because of defective activation of thromboplastic precursors. The clot retraction is usually defective. Patients with this disease have recurrent episodes of abnormal bruising and hemorrhage following trauma. The disease is hereditary, affecting both sexes.

Macroglobulinemia. In conditions in which there is an excess of plasma globulins or alteration in the relationship of plasma proteins with a relative increase in globulin or abnormal protein as in myeloma there may be hemorrhagic phenomena. It is thought that the abnormal proteins combine with and inactivate various essential coagulation components. In conditions associated with demonstrable cold agglutinins or cryoglobulin there may be precipitation of proteins in small blood vessels producing occlusion and hemorrhagic infarcts. Systemic intravascular coagulation may deplete the fibrinogen reserves and cause bleeding through this mechanism.

INTRAVASCULAR COAGULATION

The endothelial lining cells of normal blood vessels are smooth and relatively non-wettable. Thrombocytes and other formed elements of

normal blood are kept moving by the circulating blood and do no adhere to the vessel walls. Thromboplastin and thrombin, which may be formed in small amounts at sites of minor tissue trauma, are rapidly diluted and rendered inert by adsorption mechanisms and by coagulation inhibitor substances.

In the presence of more severe tissue trauma or injury of any kind, the amount of thrombin formed locally may be excessive and result in intravascular coagulation of blood in the smaller blood vessels. Clots forming in capillaries and venules may extend into larger vessels. Tissue injury in the neighborhood of a large artery or vein may first involve the blood vessels of the wall. Coagulation in such nutrient vessels causes necrosis of the media and injury to endothelial cells. Thrombocytes adhere to the endothelium at the site of injury, spread out along the surface, disintegrate and liberate activator enzyme which causes the conversion of thromboplastin precursors to thromboplastin. Prothrombin conversion to thrombin and fibrinogen conversion to fibrin follows. As the fibrin crystals develop at the site of endothelial injury, additional thrombocytes adhere and the thrombus grows and extends out into the lumen. As the clot retracts, thrombin and accelerator substances are expressed which favor further propagation of the clot. The clot sometimes extends down stream in long polypoid masses. Portions of the clot may break off, forming emboli which on reaching terminal vascular beds produce tissue infarcts.

THROMBOEMBOLIC DISEASES

Diseases associated with thrombosis and embolism are more common and are the cause of death and disability more often than are hemorrhagic diseases, but have received less attention because the symptoms are often vague, there is little visible evidence of abnormality, and the vascular occlusion is often a terminal event.

Thrombosis and embolism are usually secondary to perivascular disease, disease of the blood vessels, circulatory stasis or to alterations in the circulating blood which favor clotting. Auricular fibrillation, myocardial damage with endothelial involvement and bacterial endocarditis are often the cause of mural thrombi and embolic phenomena. Thrombosis is common in blood dyscrasias such as polycythemia, sickle cell anemia and leukemia, malignancies and infections, and vascular abnormalities such as aneurysm, thrombo-angiitisobliterans and arteriosclerosis.

Postoperative thrombosis and embolism are of major concern to the

surgeon and one of the most common causes of death following operation. The embolic phenomena may occur shortly after operation, but are more common between the first and second week after operation. Thrombosis is particularly likely to occur after splenectomy, major pelvic operations and operations on elderly and obese individuals. As a rule the thrombi start in the small veins of the leg and extend up into the larger vessels. Uterine thrombosis and emboli are common complications of the post-partum state and septic abortion.

Primary diseases due to an increase in coagulant components or to decrease in anticoagulant substances are rare. The two entities that have been described are essential thrombocytosis in which the thrombocytes are significantly increased and essential thrombophilia in which there is demonstrable decrease in the coagulation time of the blood. In both of these conditions there are multiple and recurrent thrombosis in various parts of the body.

Dicumarol and related coumarin drugs and heparin and drugs which have heparin-like activity have no effect on dissolving a clot already formed but inhibit the formation of new clots and the propagation of existing clots. Ample fluids increase fluidity of the blood and thus aid in maintenance of blood flow. Frequent change of position, forceful movements of the extremities, getting the patient out of bed soon after operation and avoidance of deep sedation aid in increasing blood flow and avoiding local ischemia. Ligation of the veins above the clot to prevent pulmonary embolism and sympathectomy to relieve vessel spasm, pain and edema have been advocated.

The routine laboratory studies are of little value in predicting the tendency toward thrombosis. The condition of blood vessel walls, spasm of blood vessels and rate of flow are of greater importance than the hemic factors. A coagulation time of less than 5 minutes, a thrombocyte count greater than 1,000,000 per cu. mm. and a clot retraction beginning within 15 minutes after the blood is drawn are presumptive evidence of impending or existing thrombus formation.

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CHAPTER 5

DISEASES PRIMARILY AFFECTING LEUKOCYTES

By

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INTRODUCTION

As a reference see "Origin and Development of Blood Cells," Plate 2.II, Chapter 2, pp. 98–99.

The examination of leukocytes in the laboratory includes:

1) two technical phases:
   a. quantitative: determines the number of all white cells (total white blood count) and the relative and absolute numbers of the various forms of white cells
   b. qualitative: determines the changes from the normal in appearance of the cytoplasm and nuclei
2) two anatomic phases:
   a. examination of peripheral blood (capillary or venous)
   b. examination of marrow (sternal, tibial, iliac, spinous process).

The purpose of the study of leukocytes is to help:

1) in establishing a diagnosis. Occasionally the examination alone may furnish a positive specific diagnosis, for example, in leukemia; more frequently it may be diagnostically helpful together with other clinical and/or laboratory data, for example, in acute appendicitis or agranulocytosis.
2) in prognosis. For example, a low white blood count in acute appendicitis or pneumonia is considered prognostically unfavorable. For further examples see p. 231.
3) in following up the course of disease. For example, toxic effects of radiotherapy and chemotherapy may be recognized early by examination of leukocytes.

Examination of leukocytes may reveal the existence of an entirely unsuspected disease. For example, leukemia may be found in a patient with the clinical picture of an acute cholecystitis, or infectious mononucleosis may be found in patients clinically resembling leukemia.

For this and other reasons the white blood count is one of the so-called routine tests which, according to recommendations of organiza-

tions supervising hospitals, have to be done on every patient admitte to the hospital regardless of disease.

THE NORMAL WHITE BLOOD COUNT

For technics employed in the study of leukocytes, see pp. 25–32 an 36–48.

It is generally agreed that a white blood count falling within th range of 5,000–10,000 per cu. mm. is within normal limits. By that i meant that a study of large numbers of what appear to be healthy indi viduals showed that 80 per cent of them had white blood counts ranging between the two values mentioned. About one person in five will have a count either higher or lower than the mentioned values. If 95 per cent of those tested were included as normals the range would be 4,000–11,000. According to the best judgment of the examiners the 20 per cent beyond the 80 per cent range showed no perceptible signs of abnormality or illness.

Some factors responsible for this wide range of normal are known but many more are not known.

For example, it is known that the condition of the host plays an important part as was shown by Garrey. According to his studies the upper limits of normal, under basal conditions, are 3,000–4,000 cells per cu. mm.

Most of the white blood cells after the first few years of life are granulocytes. They are produced in the bone marrow. Therefore, the condition of the bone marrow, and the release mechanism, both variables, have a bearing on the total number of white blood cells. It seems that

TABLE 5.1

The Normal White Blood Count

	Range	Average
	per cu. mm.	*per cu. mm.*
Newborn		
1st day	3,600–45,000	15,000
2nd day	5,000–13,000	10,000
3rd–10th days	5,000–13,000	7,500
Infants		
10 days to 7 years	5,500–15,500 (95% value)	10,400
Adolescents	4,000–13,000 (95% value)	8,400
Adults	5,000– 8,000 (80% value)	

the release of the granulocytes from the bone marrow occurs at regular intervals. The highest total count and the highest number of neutrophilic leukocytes are seen late in the afternoon. This is known as diurnal variation. The conception that food intake influences the total white count has been given up, at least as it concerns the normal individual. The influence of allergy will be discussed later.

Basal conditions and prolonged rest lower the white blood count. Muscular activity, especially if excessive, raises the total white count. As a rule, the increase is in granulocytes, sometimes in lymphocytes. It has been shown that during exercise the spleen tends to contract excessively. It is possible that this causes an increase of the peripheral white blood count by expelling the stored blood and leukocytes with it. Cold showers or massage may raise the white blood count up to 25,000. Mental factors such as pain may produce leukocytosis.

There is no evidence that sex influences the white blood count except in the last month of pregnancy, when variations from the normal seem to be exaggerated, and during labor when there is as a rule a striking increase in the total white count ranging from 15,000 to 30,000. It lasts for a day or two and returns to normal after from four days to two weeks post partum.

Age is one of the important factors influencing the white blood count, quantitatively and qualitatively. Fluctuations of white blood cells are common at all ages, but they are greatest in infants, and only slightly less so in childhood.

In the newborn baby the average count is quoted as 15,000, with occasional values as high as 45,000, with a rapid drop on the second day continuing until about the age of 10 days. Nucleated red blood cells in the newborn, especially in the premature, may cause errors in leukocyte counts if proper corrections are not made. After 10 days until the age of about 7 years the range is from 4,500 to 15,500. This range is narrowed to from 4,000 to 13,000 in the age period of from 8 to 18 years. Then the values quoted for adults prevail.

Variations similar to those for the total white blood count prevail also with regard to the relative proportion of the different white blood cells, the so-called differential blood count. According to Wintrobe the range of neutrophilic granulocytes in adults is 54 to 62 per cent. According to Osgood, in the newborn the average is 60 per cent on the first day; it drops to 38 per cent on the fifth day and continues approximately at the same level with a range of 16 to 60 per cent. In adolescents, the average increases to 48 per cent and the range to from 25 to 70 per cent.

Band cells are found in from 0 to 5 per cent of white blood cells in the

adult, as high as 25 per cent in the newborn infant during the first day of life, up to 17 per cent during the first year of life, and then slowly reach the values seen in the adult. Values over 10 per cent in the differential count are abnormal.

Eosinophils range from 1 to 3 per cent with an average of 2 per cent in adolescents and adults. The values are slightly higher in children.

Basophils are rare: they range from 0 to 1 per cent.

Normal values for lymphocytes in adults range from 25 to 33 per cent of the white blood cells. In the newborn the average is 30 per cent on the first day with a slow rise up to 45 per cent on the fourth day. Until the age of 14 years the average is 48 per cent and the range from 20 to 70 per cent. The average drops to 42 per cent and the range to from 22 to 62 per cent in adolescents.

Prevalence of lymphocytes over neutrophilic leukocytes may sometimes continue far beyond the age mentioned and into adult life. The reason for persistence of infantile lymphocytosis is unknown.

Monocytes are quoted as averaging 4 per cent of white blood cells in adolescents and adults with a range of 3 to 7 per cent; an average of 5 per cent, with a range of 0 to 12 per cent in newborn and infants up to 4 years of age; an average of 3 per cent and a range of 0 to 7 per cent in children 4 to 13 years of age.

The wide range of variations in the total white blood count and in the relative proportions of the different components of the white count makes it apparent that relative figures expressed in percentages are of limited value and frequently misleading. Therefore, it is important to learn the absolute values of these cells per cu. mm. Familiarity with and use of absolute values help to prevent erroneous conclusions. The finding of 10 per cent lymphocytes in a differential count would at first suggest reduction in the number of these cells, but assumes an entirely different aspect when it is expressed in absolute numbers of a total white count of 25,000. The absolute values are listed in Table 5.2.

THE DIFFERENTIAL COUNT

In addition to the study of the numbers of leukocytes of the three types (granulocytes, lymphocytes and monocytes)—the quantitative study, evaluation of the blood picture includes consideration of the age of the cells—the qualitative study. From this point of view most attention has been given to cells of the granulocyte series. Several methods of classifying granulocytes according to the degree of maturation have been suggested. Arneth was the first to attempt practical prognostic

TABLE 5.2

The Normal Differential Count

(95 per cent values)

	Relative values		Absolute values	
	Average	Range	Average	Range
	%	%	*per cu. mm.*	*per cu. mm.*
Neutrophilic granulocytes				
Newborn–1st day	60		9,000	
Newborn–2nd day			6,000	
5 days to 14 years	38	16–60		
8 to 14 years			3,250	
15 to 19 years	48	25–70		
Adolescents	48	25–70	4,000	1,500–7,500
Adults		54–62	4,000	3,000–5,800
Band cells				
Newborn–1st day	25			
1st year	17			
Adults		0–5		0–1,500
Eosinophils				
Under 14 years	2.8	0–8		0–600
Over 14 years	2	1–3		50–250
Basophils	0.5	0–1		0–200
Lymphocytes				
Newborn–1st day	30			
Newborn–4th day	45			
4 to 7 years	48	20–70	5,000	1,500–8,500
8 to 14 years	48	20–70	4,000	1,500–6,500
15 to 19 years	42	22–62	3,250	1,500–5,000
Adults	28	25–33	2,100	1,500–3,000
Monocytes				
All age groups			375	285–500
Birth to 4 years	5	0–12		
4 to 14 years	3	0–7		
14 years and over	4	0–9		
Disintegrating cells	5	0–12	400	0–1,200

evaluation of granulocytes by dividing them into five classes according to the number of lobes. Band cells and cells younger than they formed Class I. The other classes were determined by the presence of two, three, four and five segments. About 35 per cent of the segmented cells have two lobes, 41 per cent three lobes, 17 per cent four lobes, and not more than 2 per cent have five lobes, which is the highest number of lobes in normal granulocytes.

Arneth worked out a very elaborate but impractical system of grading. In addition to its complexity the weakness of the Arneth system was emphasis on classification of segmented cells, which are prognostically least important.

Schilling's differential count enjoys the greatest popularity. The granulocytes are divided according to their age in four groups: (1) myelocytes and cells younger than they; (2) metamyelocytes; (3) bands; and (4) segmented cells. In normal blood myelocytes are absent, metamyelocytes may be seen occasionally, the frequency of the other cells is stated in Table 5.2. In hemograms (graphic presentations of differential counts) the cells are usually listed from left to right in order of increasing maturity, therefore an increase of immature forms is spoken of as a "shift to the left."

A "shift to the left" or an increase of immature granulocytes in the differential count means inability of marrow to produce or release enough mature granulocytes in response to an excessive demand. Instead immature cells are sent out as substitutes. This myeloid or marrow insufficiency is usually temporary. The shift to left may be "regenerative" if the total count is high and mature cells are present in ample numbers, or "degenerative" if the count is normal or low, the mature cells are below normal numbers, and the nuclei of the immature cells are shrunken and pyknotic, i.e. show signs of degeneration.

In a "shift to the right" there are fewer band cells and an increase of polysegmented "aged" cells, a phenomenon seen especially in pernicious anemia.

Filamented cells are those in which segments or lobes are connected with a distinct thin filament of chromatin. The filament must be thin. Just a thinned-out portion of the nucleus is not sufficient to classify them as filamented.

Toxic granulation

Damaging action of severe infections and of other toxic conditions is manifested in so-called "toxic" granules of the cytoplasm. These are basophilic granules unevenly distributed if large, and diffusely if small. They are peroxidase positive. Damage takes place early in the development of the cell, probably at the stage of granule formation.

Basophilia of the cytoplasm and vacuolization are other signs of toxic damage.

Based upon the number of segmented granulocytes showing basophilic granules, a so-called degenerative index was proposed by Kugel and

Rosenthal. It is computed by dividing the total number of neutrophils into the total number of cells with toxic granules. For example, if the total percentage of neutrophils is 75, and 55 of them show basophilic granulation, then 73 per cent show this change and the degenerative index is 73. The higher the index the more serious is the prognosis. The quality of the stain has an important bearing on the appearance of the granulocytes. Toxic granulation may be simulated by artefacts caused by imperfect staining.

The accuracy of the differential count is directly proportionate to the numbers of cells counted and the error is increased in relation to the total white blood count unless a proportionately large number of cells is counted. In other words, the higher the total white count the larger the number of cells that must be counted to obtain an accurate differential count.

The following table was found useful:

Normal	100 cells
Leukocytosis	200 or 300 cells
Leukemias	500 cells

LEUKOCYTOSIS

Definition

Leukocytosis is an increase of the total leukocyte count above the upper normal limit of 10,000 per cu. mm.

The white blood picture is regulated by a complex mechanism which is not yet fully understood. The central nervous system is probably one of the more important factors.

Hydrogen ion concentration may be another factor. During the early phase of neutrophilia there is a tendency towards acidosis. This can be confirmed experimentally: acidosis brings about a neutrophilic leukocytosis and alkalosis a lymphocytosis. In diabetic acidosis there is a leukocytosis with a shift to the left just like in sepsis, but in alkalosis of tetany leukocytes show low values with a relative lymphocytosis.

The Relation of the Hypophysis and Adrenals to Leukocytes

In the alarm reaction of Selye, of which infection is one cause, there is an activation of the suprarenals. According to Dougherty and White, corticotropic hormone of the hypophysis causes a drop of lymphocytes in the peripheral blood and disintegration of lymphocytes in lymph nodes, spleen and thymus. This reduction of lymphocytes can be caused

not only by the corticotropic hormone of the pituitary but also by the hormones of the adrenal itself, especially 11-oxycorticosteroids. The breakdown of lymphocytes liberates globulin, especially gamma globulins, the protein fraction in which most antibodies are present.

Mobilization of Leukocytes

Leukocytes are produced in the marrow. They leave the marrow by ameboid movement when attracted to a focus of inflammation by chemotactic substances. The passage through the walls of blood vessels is facilitated by the tendency of leukocytes in circulating blood to flow along the margins when circulation slows down.

This so-called margination of leukocytes has been explained as follows: in suspensions flowing in tubes, on slowing up of circulation the heaviest particles get into the axis and the lightest go to the periphery. Leukocytes are lighter than red cells. They stick to the endothelium due to their natural stickiness which is further increased with a rise of the surface tension. The latter is brought on by the acid metabolic products of the inflammatory focus.

There are about 5 billion leukocytes in a milliliter of pus. Therefore, all the leukocytes of the circulating blood are sufficient just for 10 ml. of pus. This relative scarcity of leukocytes is further accentuated by their short life span—only about 8–10 days in circulating blood, with slight differences upward and downward, depending on the variety of the cells (granulocyte, lymphocyte, monocyte). These data have been arrived at with the help of recent studies employing leukocytes labeled with radioisotopes. In purulent foci, the life span is considerably shorter.

Physiologic Leukocytosis

(See Table 5.3)

Pathologic Leukocytosis

The term means an increase of leukocytes due to disease. As a rule it is neutrophilia, an increase of segmented granulocytes. The neutrophilia may be relative—an increase in the differential count, or absolute—an increase of the total number of the cells, or both. The latter is the more frequent finding.

Sometimes the relation of leukocytosis to disease is not easily demonstrable. Attention must be paid to the presence or absence of immature cells, of eosinophils and monocytes. Still more important is correlation with the clinical condition of the patient. A leukocyte and

differential count by itself is of little value as an aid to diagnosis unless it is related to the clinical condition of the patient. Only then is a correct and useful interpretation possible.

Infectious Leukocytosis

The characteristic pattern of infectious leukocytosis is made up of three component parts: (1) progressive neutrophilic leukocytosis (neutrophilic phase), (2) increase of young forms (shift to left), and (3) drop of eosinophils—all three reaching their peak when the infection is at its peak. When the infection begins to subside and the fever drops, a gradual transformation in the blood picture takes place: the total number of leukocytes goes down, and monocytes increase (the monocytic phase of infection). A relative lymphocytosis (lymphocytic phase) and an increase of eosinophils are signs of advancing recovery.

The neutrophilic phase represents the period of active resistance to infection. When the process of defense is finished and the peak is passed the monocytic phase follows, and the end phase or the phase of recovery is lymphocytic. One could also say that the fight against the infection is characterized by neutrophilia, overcoming of the infection by monocytosis, and the healing by lymphocytosis.

Leukocytic, monocytic, and lymphocytic reactions may occur without infection in a variety of circumstances such as severe muscular exertion, menstruation, during anesthesia and operations, following perspiration in baths at high temperature, following exposure to high temperatures in hyperthermia, and in response to stimulation by foreign substances. Similar blood changes may accompany tissue breakdown when its products are not disposed of in the usual physiologic way. The same may be seen in some diseases of the central nervous system and following experimental irritation of the central nervous system.

Etiologic Classification of Neutrophilia:

1. Infectious Neutrophilia. *a. General infections.* Systemic infections caused by various bacteria, fungi, spirochetes, and viruses. In some of these conditions the leukocytosis may be preceded by a transient initial leukopenia, particularly if the infection is overwhelming. It is thought that the severe damage uses up rapidly the available leukocytes and the leukopenia persists until the marrow can meet the demand. On the other hand there are bacterial infections which exert a depressing effect on the marrow, especially on its granulocyte producing or releasing ability with resulting leukopenia. Here belong: typhoid fever, para-

TABLE 5.3

Physiologic Leukocytosis

Definition: increase of leukocytes due to factors other than disease or injury

Cause	Cell type	Mechanism
Acute anoxia	Neutrophilic granulocytes	
Adrenalin	Neutrophilic granulocytes and lymphocytes	Contraction of spleen. Occurs also after splenectomy
Artificial fever	Neutrophilic granulocytes (may be preceded by leukopenia)	
Cold showers, massage	Neutrophilic granulocytes	Emptying of depots. Accelerated blood flow
Dehydration	Neutrophilic, eosinophilic and basophilic granulocytes, lymphocytes, monocytes	Concentration
Emotional disturbance, excitement, pain, nausea, vomiting	Neutrophilic granulocytes	Nervous mechanism. Accelerated blood flow
Ether anesthesia	Neutrophilic granulocytes	Struggling during excitation may be responsible
Ether spray of skin	Neutrophilic granulocytes	
Exercise	Neutrophilic granulocytes or lymphocytes	Accelerated blood flow. Emptying of depots (spleen, liver, lungs, etc.), but occurs even after splenectomy. Increased adrenalin output
Labor	Neutrophilic granulocytes	Emptying of depots. Accelerated blood flow
Newborn state	Neutrophilic granulocytes	
Sunlight: ultraviolet irradiation	Lymphocytes	

There are as a rule no immature neutrophilic granulocytes in the circulating blood in leukocytosis of this type (no significant shift to left) and the eosinophilic leukocytes and monocytes do not disappear from the blood as they do in leukocytosis due to infections.

TABLE 5.4

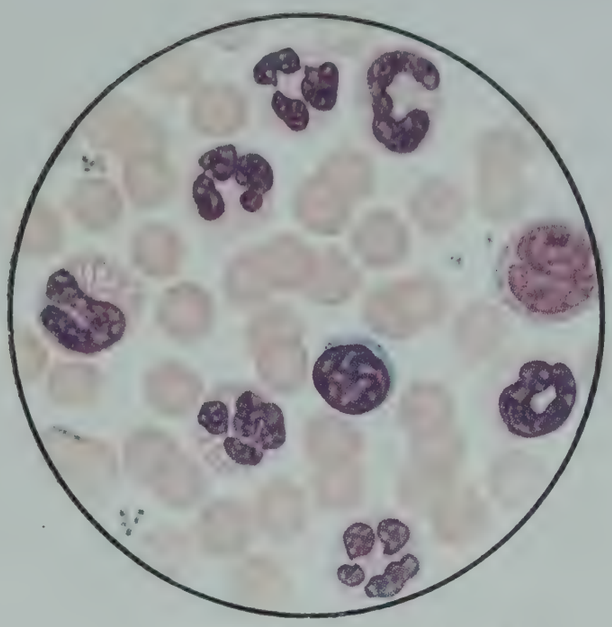
Pathologic Leukocytosis

Cause	Cell Type
Allergy	Eosinophil
Brucellosis	Lymphocyte, monocyte
Convulsions	Neutrophilic granulocyte or lymphocyte*
Drugs and poisons:	
camphor	Neutrophilic and eosinophilic granulocyte
copper sulphate, phosphorus, pilocarpine	Eosinophilic granulocyte
tetrachlorethane, adrenalin	Monocyte, neutrophilic granulocyte and lymphocyte
other (acetanilid, arsenicals, benzol, CO, digitalis, lead, phenacetin, turpentine, venoms)	Neutrophilic granulocyte
Erythremia	Neutrophilic, eosinophilic, basophilic granulocyte
Hemolysis	Neutrophilic granulocyte
Hemorrhage	Neutrophilic granulocyte
Hodgkin's disease	Neutrophilic and eosinophilic granulocyte, monocyte
Infectious lymphocytosis	Lymphocyte
Infectious mononucleosis	Lymphocyte
Loeffler's syndrome, periarteritis nodosa, pernicious anemia	Eosinophilic granulocyte
Toxemias:	
diabetic acidosis, eclampsia, gout, uremia	Neutrophilic granulocyte
Tuberculosis	Neutrophilic and eosinophilic granulocyte, lymphocyte, monocyte
Tumors, involving:	
marrow and serous cavities	Neutrophilic and eosinophilic granulocyte
ovarian tumor	Eosinophilic granulocyte
G-I tract and liver	Neutrophilic granulocyte
Typhoid fever	Lymphocyte

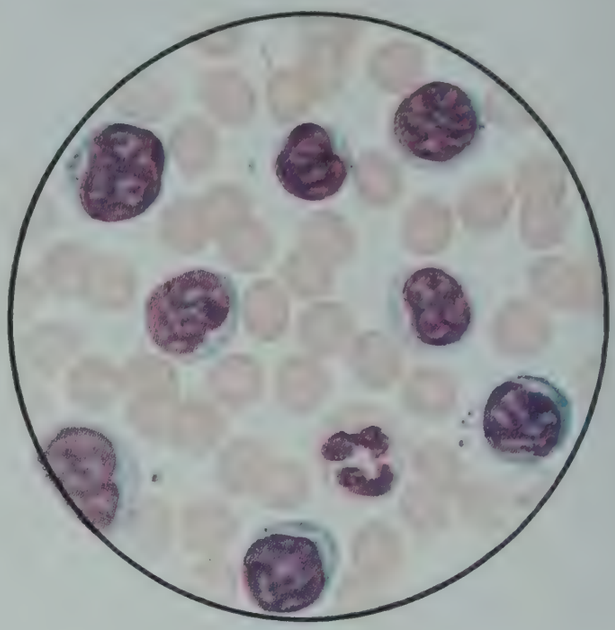
* Pathogenesis: 1. Emptying of depots (spleen, liver, lungs, etc.) but occurs even after splenectomy. 2. Increased adrenalin output.

PLATE 5.I

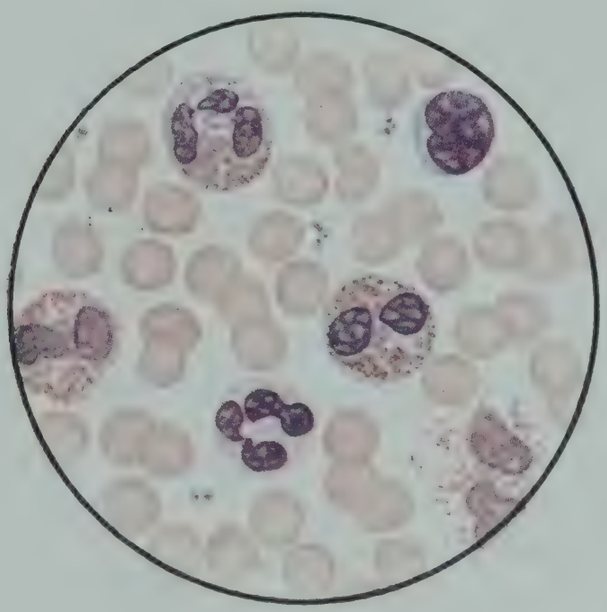
VARIOUS TYPES OF LEUKOCYTOSIS



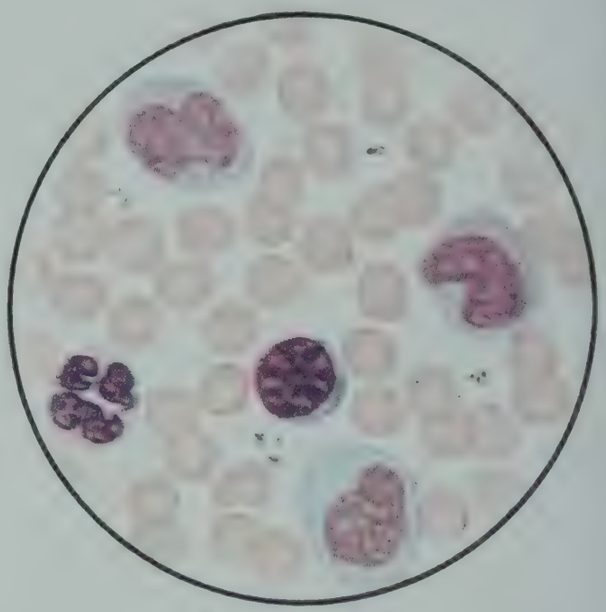
Neutrophilic Leukocytosis



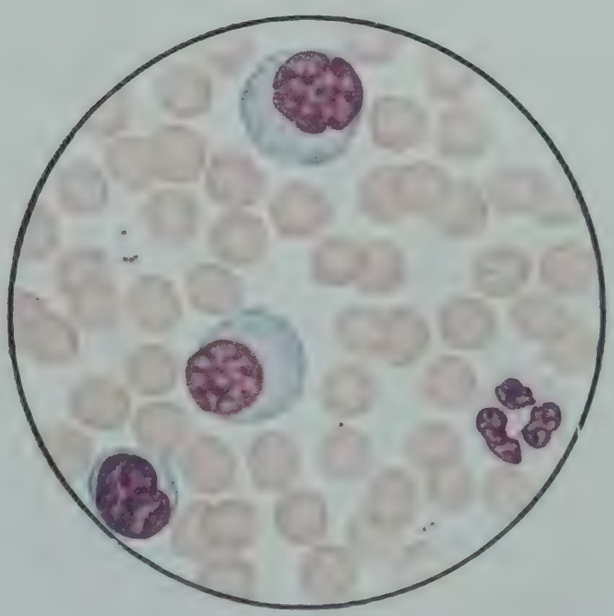
Lymphocytosis



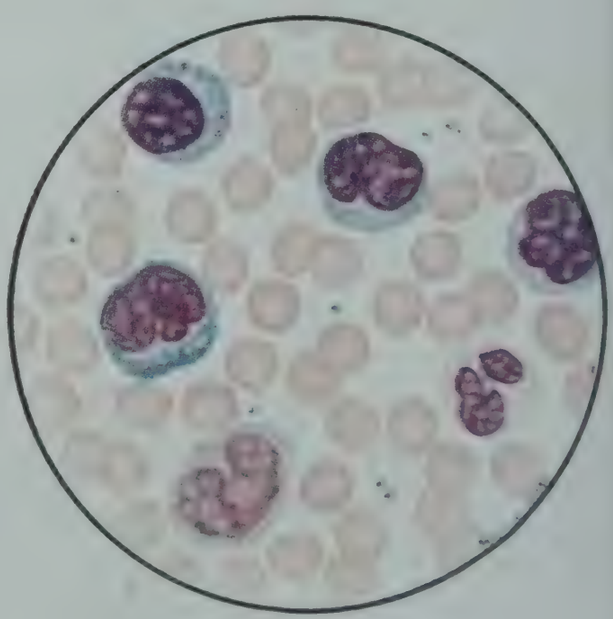
Eosinophilia



Monocytosis



Plasma Cell Reaction



Infectious Mononucleosis

typhoid fever, and tuberculosis, except when complicated by a secondary infection or in special forms as tuberculous meningitis, peritonitis and pleuritis.

b. Local infections. Here belong appendicitis, salpingitis, otitis media, mastoiditis, and various other circumscribed accumulations of pus, usually caused by one of the pyogenic bacteria, such as streptococci, staphylococci and pneumococci. The size of the abscess is less important than the pressure exerted in it. The effect on the blood picture is lessened with organization of a limiting pyogenic membrane.

2. Toxic Neutrophilia. *a. Endogenous intoxications:* uremia, eclampsia, gout, diabetic acidosis, burns. A chemotactic substance attracting granulocytes from the marrow or a substance specifically stimulating the marrow may be responsible. In burns it may be absorption of denatured proteins. In eclampsia convulsions may be a contributory factor.

b. Drugs and poisons: various chemicals and drugs such as lead, mercury, potassium chlorate, digitalis and adrenalin, and many others. Among them are chemicals which under certain circumstances are known to produce severe neutropenias (benzol, sulfonamides, arsphenamine).

3. Posthemorrhagic Neutrophilia, especially if hemorrhage took place into a serous cavity (peritoneum, pleura, joint, subdural space). Here belongs also leukocytosis following a hemorrhage into the lumen of an intestine.

Leukocytosis following intracranial hemorrhage is probably at least in part caused by increased intracranial pressure. In the other posthemorrhagic forms rapid absorption of a chemotactically active substance of the blood is incriminated.

4. Neutrophilia Due to Tissue Destruction. Here belongs neutrophilia following injections of turpentine; in myocardial infarction; after operations; in cancer of liver, gastrointestinal tract, or bone marrow, especially with extensive necrosis; in burns; after rapid hemolysis of red cells; in hemolytic crises of hemolytic anemia and in hemolytic transfusion reactions.

Determinants of Neutrophilic Leukocytosis:

A. Host Factors. *1. Age.* Children respond more intensely and more rapidly to a leukocytogenic stimulus than adults.

2. Resistance. An ill-defined property frequently highly specific for a certain micro-organism.

3. Marrow efficiency or reserve. Ability of the marrow to respond rapidly and efficiently to leukocytogenic stimuli.

4. Localizing ability of body in response to invasion by infectious agents. The more localized the process the more pronounced is the neutrophilia, and vice versa.

B. The Infectious Agent. *1. Specific leukocytogenic properties of micro-organism*, especially of pyogenic bacteria.

2. Virulence of micro-organisms. There is within limits a direct relationship between virulence of the infecting micro-organism and the degree of neutrophilia. This property is frequently conditioned by the spreading ability or invasiveness of the micro-organism, as opposed to the localizing ability of the host.

A highly virulent micro-organism endowed with a high degree of invasiveness may produce only a mild neutrophilic leukocytosis even with a severe infection.

It is claimed, and it is probably correct within wide limits, that the height of leukocytosis is an indicator of the resistance of the individual, and that the degree of the shift to the left is an indicator of the severity of the infection. In keeping with this conception a simultaneous fall of the former and a rise of the latter is prognostically unfavorable.

Prognostic conclusions in the study of leukocytes can be drawn from:

(1) quantitative data
  (a) height of leukocyte count
  (b) decrease in number of eosinophils
  (c) number of lymphocytes (see Hodgkin's disease)
  (d) number of monocytes in relation to lymphocytes (see tuberculosis)
(2) qualitative data
  (a) shift to left
  (b) toxic granulation of leukocytes

Hematologic signs of recovery from infectious diseases:

(1) drop of the total leukocyte count and of neutrophils
(2) disappearance of shift to the left
(3) increase in number of monocytes (except in tuberculosis, see p. 256)
(4) increase of eosinophils where they were decreased or absent during the height of the disease
(5) increase in number of lymphocytes
(6) disappearance of toxic forms

Therefore, the following are hematologic unfavorable signs:

(1) a moderate or slight rise in the total number of leukocytes associated with a marked shift to the left during the height of the disease
(2) failure of eosinophils to reappear in the end stages of an infectious disease where they were absent before
(3) absolute reduction of lymphocytes
(4) excessive number of cells with toxic granulation

Specifically in tuberculosis, in addition to the increase of monocytes, the disappearance of young leukocytes is prognostically bad. Therefore, improvement is indicated by a drop of monocytes and reappearance of young leukocytes and increase in the number of lymphocytes.

MARROW

The total weight of the marrow in the adult is about 2,600 Gm. which is more than any other organ except circulating blood, skeletal muscles and bones. Approximately one-half of marrow is functioning, red marrow; the remainder is space-occupying yellow, fatty marrow.

Fat marrow may at any time be replaced by functioning marrow. This change may occur at an astoundingly rapid rate, especially in the young. That explains the extreme changes that may be seen in the peripheral blood on slight provocations, especially in the young.

Anatomy

Age. In the newborn only red marrow is present. With growth, recession of red marrow takes place from distal ends of extremities (centripetal). In the normal adult red marrow is found only in bones of trunk, skull, and proximal ends of femur and humerus. In hyperplasia the transformation of fat to red marrow progresses in centrifugal direction.

Growth. Erythrocytes grow in intersinusoidal capillaries, which can open to the general circulation but which normally are closed until the cells reach a certain stage of maturation. When that is reached, the capillary reopens into general circulation and the cells are thrown out. They are thrown out as reticulocytes, of which there is normally about 1 per cent. It is probable that in cases of special need—for example, loss of blood in large quantities—the release or the washing out of the intersinusoidal capillaries takes place before the cells mature. The formation of granulocytic cells is extrasinusoidal. It is assumed that they get into the circulation by ameboid motion. This is not present in myeloblasts and progranulocytes but is present in increasing degrees in

myelocytes, metamyelocytes, etc. For nomenclature of blood cells see Table 2.2 (p. 93) and Table 2.3 (p. 95).

Marrow Changes

Hyperplasia. Definition: increase of cellular marrow at expense of fat marrow. In extreme degrees even bone may be replaced by proliferating marrow cells (osteoporosis).

Hyperplasia may involve all marrow components (general hyperplasia) or only one (for example, granulocytic hyperplasia), or any combination of them. Hyperplasia may also be generalized and involve most or many bones, or local, limited to one region or bone. Hyperplastic marrow may consist of fully differentiated cells or of cells arrested at various stages of maturation (arrest of maturation). The peripheral blood may reflect the hyperplasia of marrow by showing a corresponding increase of fully differentiated or immature cells (marrow hyperplasia with normal marrow blood threshold), or the hyperplasia of the marrow is not manifest by a corresponding increase in the circulating blood (marrow hyperplasia with elevated marrow blood threshold).

Hypoplasia. Definition: decrease of cellular marrow and replacement by fat, connective tissue, bone, metastatic cancer, malignant lymphoma, Hodgkin's disease, multiple myeloma, etc. Hypoplasia of marrow may or may not be accompanied by pancytopenia.

All elements of marrow may be affected (general marrow hypoplasia) or only one or any combination of them. Excessive hyperplasia of one or more marrow elements may be responsible for hypoplasia of the others. For example, in granulocytic leukemia, the granulocytes may be hyperplastic to an extent interfering with the normal growth of red blood cells and megakaryocytes with resulting anemia and thrombocytopenia.

Aplasia. Definition: extreme degree of general marrow hypoplasia.

EOSINOPHILIA

Definition

Eosinophilia (Plate 5.I) is a rise of eosinophilic granulocytes above the highest normal of 500 per cu. mm. counted just like white blood cells with a special diluent.

The close association of eosinophilic leukocytes with allergy, broadly conceived, is impressive. One could easily correlate practically all eosinophilias with one form of allergy or another. An individual host factor has to be assumed to explain variations in eosinophil response.

The eosinophils in the blood come from the marrow, those in the tissues come from the blood.

Conditions Characterized by Eosinophilia

1. Allergic diseases: bronchial asthma, hay fever, angioneurotic edema, urticaria, erythema multiforme. Eosinophils are found in the peripheral blood, marrow, sputum (in bronchial asthma), nasal and conjunctival discharges (in hay fever), in the urticarial skin lesions and vesicles. Blood eosinophilia is usually only mild or moderate.

2. Skin disorders of various kinds. In some of them the allergic background is apparent, for example, allergic eczema, dermatitis venenata; in others it may be surmised. There is frequently a clear-cut direct relation between the degree of eosinophilia and extent of cutaneous involvement.

3. Parasitic infestations. Eosinophilia is more pronounced if tissues are invaded (for example, trichinosis) than when parasites are inhabiting the lumen of a viscus (for example, tapeworm). The role of free exchange of tissue fluids (metabolic continuity) is evident by disappearance of eosinophilia in some forms of infestation when encystment occurs (for example, cysticercosis).

Neutrophilia depresses eosinophilia. This is well shown in the disappearance of eosinophilia when a lesion which is responsible for eosinophilia (for example, echinococcus cyst) becomes infected, suppurates and is followed by neutrophilia.

The same phenomenon is also observed in acute infections (for example, pneumococcus pneumonia).

Eosinophilia may be absent in most severe infestations with trichinae. The prognosis in such cases seems aggravated.

4. Eosinophilia of various degrees is seen in many infectious diseases. Some of them (for example, scarlet fever) have cutaneous rashes, probably of allergic nature; some (like brucellosis) have granulomatous lesions with obvious allergic overtones. It is possible that the blood and tissue eosinophilia of Hodgkin's disease is of similar nature.

It is in infectious disease that the depression of eosinophilia by neutrophilia is particularly noticeable.

Chorea may show eosinophilia, though other forms of rheumatic fever do not.

5. Alimentary eosinophilia. Relation to food allergy is suggestive. It is seen in patients with pernicious anemia. The claim that liver

therapy is responsible is negated by reports of eosinophilia in patient with pernicious anemia before the era of liver therapy.

In so-called allergic diarrhea there is a large number of eosinophil in the mucous discharge.

6. Blood diseases. In chronic granulocytic leukemia general marrow hyperplasia may be responsible for the eosinophilia as well as for the basophilia. Eosinophilia may be seen in pernicious anemia to be replaced by eosinopenia in late stages. Marrow eosinophilia may be present in thrombocytopenic purpura. Eosinophilic leukemia is discussed with other leukemias.

7. Splenectomy is frequently followed by eosinophilia and lymphocytosis. Neutrophilia, if previously present, recedes. This may last for several months. On the other hand, injection of splenic extracts in animals has been reported to produce eosinopenia. This suggests that hyposplenism may favor eosinophilia, and hypersplenism eosinopenia.

8. The constitutional element in eosinophilia is exemplified in familial eosinophilia. In some reported cases infestation with parasites and subclinical allergy have not been excluded.

9. There is no satisfactory explanation for occasional instances of moderate and even severe eosinophilia, general and/or local in patients with various neoplasms, and a variety of other conditions (for example, ovarian cysts, etc.). Eosinophilia is seen more frequently in neoplasms involving serous surfaces and bone and in those with excessive necrosis.

10. Various drugs have been reported as responsible for eosinophilia: pilocarpine, physostigmine, digitalis, phosphorus, benzol, parenterally administered liver extracts and insulin, etc. On the other hand, atropine is supposed to depress the eosinophils.

11. Local tissue eosinophilia, with or without blood eosinophilia: here belong eosinophilic granuloma, polyps of nose, chronically inflamed pleura, and Loeffler's syndrome (a pulmonary lesion). The latter has been ascribed to Ascaris infestation, at least in some cases.

EOSINOPENIA

Definition

Eosinopenia is a drop below the lowest normal of 45 per cu. mm. It is seen most frequently in the presence of infectious neutrophilia, in severe infections. In Cushing's disease the count ranges from 0–30 per cu. mm. It results from hyperactivity of the adrenal cortex. After major surgical operations there is a drop within 4–6 hours in the presence of adequate adrenal function. In postoperative shock, not associated

with massive hemorrhage, normal or high eosinophil count indicates adrenal insufficiency.

Eosinopenia is seen after electric shock therapy, in eclampsia, and in labor.

The eosinopenia following parenteral administration of certain adrenal cortical hormones and of substances (ACTH and epinephrine) which increase the output of these hormones is the basis of a test for adrenal function.

Thorn recommended a test for evaluation of adrenal cortical function, based on the development of an eosinopenia following the injection of ACTH (adreno-corticotropic hormone). Absence of a significant drop of eosinophiles is interpreted as evidence of adrenal cortical insufficiency. At the present time, the value of the test is limited.

Technic of Absolute Eosinophil Count

Regular or special (Fuchs-Rosenthal) counting chamber and a special staining fluid are used (Randolph).

The technic of counting white blood cells described on pp. 25–32 applies to counting of eosinophiles.

BASOPHILIA

Definition: increase of basophilic granulocytes in the peripheral blood beyond the normal range of 0–1 per cent.

Basophilia is seen most frequently in chronic granulocytic leukemia, myeloid metaplasia (extramedullary myelopoiesis), and polycythemia. Relative basophilia may be transient following irradiation.

Tissue basophils, mast cells, are entirely different from basophilic granulocytes.

BASOPENIA

In view of the rarity of these cells in normal persons, a decrease cannot be readily detected and interpreted except in acute infections, when basophils disappear at first together with the eosinophils and reappear later with the lowering of the neutrophilia.

LYMPHOCYTOSIS

Definition

Lymphocytosis (Plate 5.I) is an increase in the number of lymphocytes in the peripheral blood above the normal range of from 1,500 to 3,000 per cu. mm. in the adult and of from 1,500 to 8,500 in the child.

It is present in various infections, but mainly late in the disease and during recovery. It is common in exanthems (for example, German measles); also reported in brucellosis, secondary and congenital syphilis. It is considered a good omen in tuberculosis.

In pertussis, lymphocytosis may be high early in the catarrhal stage and persist all through the entire course and late in convalescence.

Infectious Lymphocytosis

An infectious and contagious disease, it occurs mainly in children and has an incubation period of from 12 to 21 days, sometimes without systemic manifestations, sometimes with vomiting, fever, abdominal discomfort, signs suggesting involvement of the nervous system, cutaneous rashes and upper respiratory infections. Usually the high white count (from 20,000 to 40,000 and higher) precedes the clinical manifestations. The cells are mature, adult, small lymphocytes, not like those seen in infectious mononucleosis. The lymphocytosis lasts from 3 to 5 weeks and longer. There are no other blood changes. Bone marrow is not characteristic. Increase of lymphocytes has been observed, but is probably due to admixture of peripheral blood. Lymph node enlargement and splenomegaly are rare. Lymph node biopsy: reactive follicular hyperplasia has been noted but no characteristic changes. The presumptive and differential tests for infectious mononucleosis are both negative. For details regarding tests for infectious mononucleosis see pp. 249–255. In some cases there was an increase of white cells in the cerebrospinal fluid with about 40 per cent lymphocytes. It is distinctly infectious and contagious. The course is benign.

INFECTIOUS MONONUCLEOSIS

Definition

Infectious mononucleosis is an acute self-limited infectious disease of the reticulo-endothelial tissues, especially of the lymphatic tissues, with characteristic clinical, hematologic, pathologic and specific serologic changes.

The main reason for interest in infectious mononucleosis is the fact that it imitates many diseases, some of them serious and some inviting surgical intervention.

Clinic

Onset: vague, indefinite, similar to the onset of other infectious diseases. This stage lasts about 4 to 5 days.

Duration: The disease proper lasts, as a rule, from 7–21 days. Fever varies from mild to moderate, occasionally up to 106°F.

Other Symptoms: chills, sweats, headache, dizziness, malaise, pharyngitis, tonsillitis, retro-orbital aching, irritability, prostration, and asthenia, all of varying severity.

Lymph Nodes: enlargement may vary from slight to marked but is usually not too excessive, average about the size of a plum. The enlargement may be simultaneous with onset of fever, may follow or even precede it. It develops very rapidly, sometimes in a matter of hours. There is no perilymphadenitis. As a rule there is tenderness, but it is transient. It is important to look for it because it differentiates this type of lymph node enlargement from the one seen in leukemia.

Location: as a rule, cervical lymph nodes are involved first on one side then on the other, and then other regions are affected, including mediastinal, inguinal, etc. The enlargement has a tendency to persist for a long time.

There is frequent splenomegaly of varying degrees. Occasionally the spleen may be enlarged without noticeable lymph node enlargement. It is frequently tender and the enlargement sometimes persists for a long time.

Liver is less frequently enlarged than spleen and occasionally quite tender.

The various **complications,** the anatomic lesions underlying them and the resemblance to various diseases are all shown in Tables 5.5, 5.6 and 5.7.

Relapses are common. Occasionally there may be several and some may be more severe than the original attack.

Recurrences are rare, but they do occur sometimes after intervals of months or even years.

Mortality occurs as a rule only from complications, for example: ruptured spleen, involvement of the central nervous system, cerebral hemorrhage, complicating septic infection.

Sequelae: prolonged state of exhaustion. Other rare complications are listed in Table 5.7.

Contagiousness of the usual sporadic form is probably very slight. Epidemics do occur and many have been described sometimes of very large proportions, in schools, homes for children, etc.

Age: has been observed in patients from 3 months to 70 years but the disease occurs rarely above college age. Most epidemics have been in adolescents and children.

TABLE 5.5

Some Differential Diagnostic Problems in Infectious Mononucleosis

Signs and Symptoms	Anatomic Lesions	Resemblance to
Sore throat	Ulcerative and/or membranous pharyngitis	Diphtheria
Painful and stiff neck; sometimes convulsions and coma may be present	Rapidly enlarged retrocervical lymph nodes. Acute hyperplastic lymphadenitis. May be pleocytosis in cerebro-spinal fluid	Meningitis (serous meningitis may be present)
Generalized lymphadenopathy	Acute hyperplastic lymphadenitis	Leukemia
Abdominal pain and tenderness	Rapidly enlarged abdominal lymph nodes	
right lower quadrant		Acute appendicitis
left upper quadrant	Acute splenomegaly. Acute diffuse hyperplasia	Acute pleuritis; perinephritic abscess
acute tenderness and pain in right upper quadrant	Acute diffuse hepatitis; periportal infiltrations; enlarged lymph nodes around common bile duct	Acute hepatitis, especially if jaundice present
acute general abdominal pain, followed by shock	Ruptured spleen	Acute abdominal emergency
Cough (resembling whooping cough)	Enlarged mediastinal lymph nodes	Pertussis; Hodgkin's disease; tuberculosis
cutaneous rashes		Exanthematous disease (measles); scarlet fever especially if angina present; secondary syphilis, especially if enlarged inguinal lymph nodes and positive test for syphilis present
Puffiness around eyes	Swelling of retrobulbar tissues	Trichinosis
Toothache	Acutely swollen submandibular lymph nodes	Pulpitis
Hematuria	Specific infiltration of renal parenchyma and/or purpuric renal hemorrhage	Acute glomerulonephritis

TABLE 5.6

Why Does Infectious Mononucleosis Simulate So Many Diseases?

Clinical Findings in 106 Cases of Infectious Mononucleosis

	Number of Cases	Per Cent
Lymphadenopathy	101	95.3
Fever	93	87.7
Pharyngitis	64	60.4
without membrane	50	47.2
with membrane	14	13.2
Splenomegaly	51	48.1
Headache	26	24.5
Hepatomegaly	24	22.6
Prostration	11	10.4
Emesis	10	9.4
Pain in abdomen	8	7.6
upper abdomen	6	5.7
lower abdomen	2	1.9
Stiffness or pain in neck	6	5.7
Skin rash	5	4.7
Epistaxis	3	2.8
Icterus	3	2.8
Loss of weight	2	1.9
Diarrhea	2	1.9
Arthritic pains	2	1.9
Purpura	2	1.9
Gingivitis	2	1.9
Convulsions	1	0.9
Toothache	1	0.9
Albuminuria	14	13.2
Positive test for syphilis	3	2.8
Relapses (17 days to 2 months)	7	6.5
Recurrence (1 year)	1	0.9

In addition, the following clinical findings have been recorded in the literature:

Hematuria	Myositis or myalgia
Hemoptysis	Uterine bleeding

Sex: males more frequently affected than females (ratio 2:1).

The greater frequency of identification of the disease among nurses, college and medical students is probably explained by better medical supervision.

The disease has been described in all parts of the world and in all races.

TABLE 5.7

Diseases Simulated by Infectious Mononucleosis

I. Diagnoses Made in Our Series (106 Cases) Prior to Blood Count & Serologic Tests	*No. of cases*	II. Additional Diagnoses Recorded in the Literature
1. Agranulocytosis	1	1. Angioneurotic edema
2. Appendicitis	1	2. Asthma
3. Brucellosis	2	3. Bacterial endocarditis, subacute
4. Diphtheria	4	4. Chickenpox
5. Duodenal ulcer	1	5. Encephalitis
6. Gastroenteritis	1	6. Erysipelas
7. Hodgkin's disease	2	7. Erythema multiforme
8. Infectious hepatitis	3	8. Erythema nodosum
9. Influenza	7	9. Glottis edema
10. Leukemia	4	10. Hyperthyroidism with lymphocytosis
11. Measles	1	11. Infectious lymphocytosis
12. Meningitis	1	12. Myocarditis (abnormal ECG)
13. Nephritis	1	13. Mumps
14. Pharyngitis	7	14. Obstructive jaundice
15. Pharyngitis, ulcerative	2	15. Poliomyelitis
16. Pneumonia	1	16. Rheumatic fever, acute
17. Pneumonia, virus	4	17. Stomatitis, herpetic
18. Purpura	2	18. Syphilitic cervical adenitis
19. Scarlet fever	1	19. Syphilis, secondary
20. Serum disease	2	20. Thrombocytopenic purpura
21. Sinusitis, frontal	1	21. Trichinosis
22. Streptococcus sore throat	5	22. Tuberculosis, miliary
23. Tonsillitis, acute follicular	3	
24. Tonsillitis, acute ulcerative	1	
25. Tuberculous lymphadenitis	1	
26. Vincent's angina	4	
	63	

Serious complications of infectious mononucleosis

Rupture of spleen
Laryngeal and glottis edema
Nasopharyngeal hemorrhage
Guillain-Barré syndrome
Thrombocytopenic purpura
Hemolytic anemia
Congenital malformation of heart following disease of mother during first trimester

Blood Findings

There are no changes in the red blood cells and in platelets, except in extremely rare cases of complicating hemolytic anemia and thrombocytopenia. There is an increase of white blood cells ranging from 12,000 to 25,000, sometimes higher. The highest counts in adults that have been observed were 40,000 but counts as high as 70,000 have been seen in children. The increase is due to a rise in lymphocytes. These cells show characteristic changes which can be summed up as follows:

1. Hyperplasia or an increase in their number;
2. Hypertrophy or an increase in size of the individual cells;
3. Nuclear changes characterized by an increased density of the chromatin and by changes in shape. (Plate 5.I.) They show deep indentations; many are lobated, resembling in general outlines nuclei of monocytes. The cytoplasm shows basophilia, a decrease of azurophilic granules, and frequently marked vacuolar degeneration. Some cells resemble plasma cells—some of them with nucleoli. Some cells resemble monocytes, monocytoid deviation. Occasionally cells show anaplasia and resemble prolymphocytes. It is in these cases that one has to consider lymphocytic leukemia in the differential diagnosis.

The polymorphism of the lymphocytes is the striking feature of the differential blood picture (Fig. 5.1).

There are not infrequently rapid and as a rule transient rises in the number of monocytes. The term mononucleosis refers to an increase of lymphocytes and not of monocytes. From 60 to 90 per cent of the cells in the differential white count are lymphocytes. Usually the mononucleosis persists for weeks but may persist for months and years.

The cytologic changes are by no means pathognomonic for infectious mononucleosis. Similar cells in similar numbers may be found in other diseases, especially in upper respiratory infections in children and in liver diseases.

Cerebrospinal Fluid

Pressure is slightly increased. There is frequently pleocytosis—as a rule, only moderate—but cell counts as high as 1,000 per cu. mm. have been seen. All the cells in the cerebrospinal fluid are mononuclear. There is also an increased globulin.

Other Laboratory Findings

Serologic Tests for Syphilis. Occasional positive results have been observed, usually only transient and becoming negative after a few weeks.

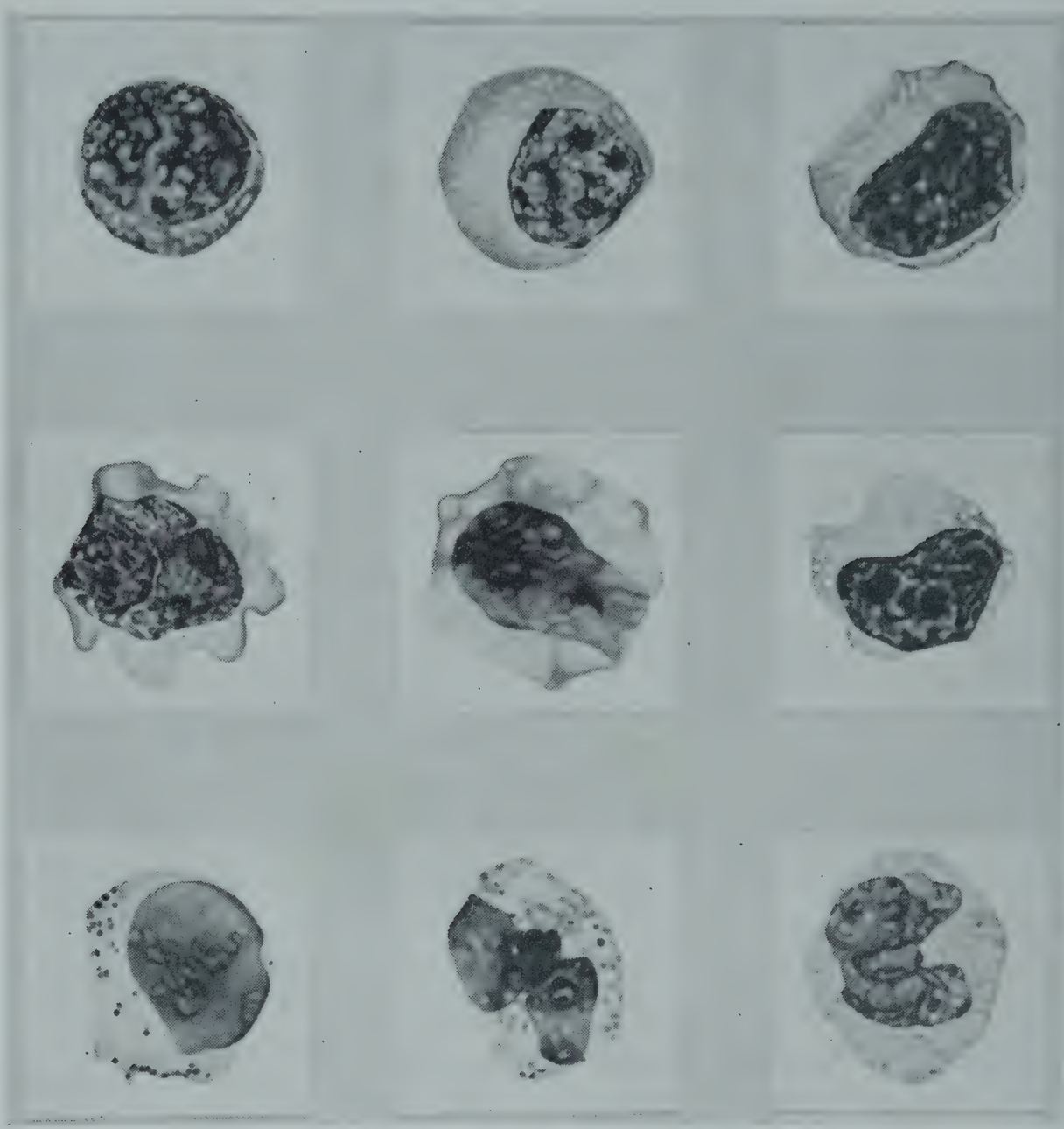


FIG. 5.1. Infectious mononucleosis

In the urine, traces of albumin may be found.

Liver function tests reveal some abnormality almost in every case. Thymol turbidity, cephalin flocculation, icterus index and alkaline phosphatase values show pathologic findings in a frequency corresponding to the order of enumeration. Numerous studies on liver function have demonstrated persistence of abnormal findings even after most clinical symptoms have almost completely disappeared; hence hepatic involvement may be responsible for the prolonged lassitude frequently found in patients recovering from infectious mononucleosis.

Pathology

Recent studies have shown extensive changes, not only in lymph nodes, but also in the spleen, liver and in other tissues which were

examined. In the lymph nodes there is a complete transformation of the architecture with filling-out of the parenchyma with proliferating large cells resembling reticulum cells. Some of them have a striking resemblance to the polymorphous cells seen in the peripheral circulation.

In the spleen, similar changes are present. The follicles are small and compressed by the proliferating cells. The capsule and trabeculae are thinned out and eaten away by the proliferation. This explains the ease with which the spleen ruptures on slight trauma or increase of intra-abdominal pressure. In many places there are subintimal infiltrations in the trabecular veins, and one can see masses of cells pouring out from the infiltration into the lumen of the blood vessels. In the liver, massive infiltrations are present in the periportal spaces, consisting of large cells resembling those seen in the spleen, lymph nodes and in the peripheral blood. Sometimes there are also infiltrations within the sinusoids.

In cases with pulmonary lesions which are sometimes seen in the disease, there are present massive peribronchial infiltrations which explain some of the changes seen in the x-ray and lead to the diagnosis of virus pneumonia (Arendt).

Occasionally large masses of infiltrating mononuclear cells are seen within the alveoli in pneumonia-like fashion.

Similar infiltrations as in other organs have also been seen in the kidneys, in various parts of the central nervous system, in peripheral nerves, etc.

The lesions in the liver explain the abnormal liver function tests which have been described recently. In our own experience the thymol turbidity test has been almost uniformly positive.

Serologic Tests

There are two tests for infectious mononucleosis (Davidsohn, a, b, c, g, h, i, j).

1. The Presumptive Test. It is based on an increase of antisheep agglutinins. Such agglutinins are present, in low titers, in the blood of most people. Their normal titer is as a rule less than 1:112. The titer is elevated in serum disease, sometimes to high levels, and less strikingly in a variety of infections, especially those of the upper respiratory system. The test is non-specific and only quantitative. If positive, in a titer of 1:224, it may be considered as confirming the diagnosis of infectious mononucleosis if the clinical and hematologic findings are characteristic for the disease.

TABLE 5.8

The Presumptive Test for Infectious Mononucleosis

Principle

The test is based on the agglutination of sheep red cells by the serum of patients with infectious mononucleosis

Technic

The physician: Obtain 5–10 ml. of blood under aseptic precautions.
Send to the laboratory.

The laboratory:

(1) Reagents:
  (a) Serum inactivated for 30 minutes at 56°C.
  (b) 2% suspension of sheep red cells

(2) Procedure:
  To 0.25 ml. of increasing dilutions of serum add 0.1 ml. of the sheep cell suspension

(3) Reading of results:
  LET STAND AT ROOM TEMPERATURE. When speed is indicated reading may be done after 15 minutes. If the result is positive (agglutination in dilutions 1:224 or higher) the test may be considered completed except that the final titer will be higher after two hours incubation. If negative (titer less than 1:224), repeat reading at intervals as frequent as convenient. Final negative result (titer less than 1:56) should NOT be recorded until after two hours incubation. If speed is not a factor, the results may be read only once, at the end of two hours.

  Results are read after shaking the test tubes to resuspend the sediment. Check with the naked eye. If no clumping is visible, place the tube horizontally on the stage of the microscope and read with a low power objective (scanning lens, e.g., 25 mm., or 35 mm.) permitting viewing of a test tube.

  The titer is the reciprocal value of the highest serum dilution still showing agglutination.

Details regarding the presumptive test are summarized in Tables 5.8, 5.9, 5.10.

2. The Differential Test. Principle: absorption of serum with a suspension of a Forssman antigen (guinea pig or horse kidney) removes antisheep agglutinins in the serum of patients with serum disease and in various other infections but not in the serum of patients with infectious mononucleosis. Here a substantial part of the antibodies remains after absorption. On the other hand, absorption with a suspension of beef cells removes the antisheep agglutinins in infectious mononucleosis but leaves them in a certain proportion of other bloods. It is this combination of no or incomplete removal of antibodies with Forssman antigen

TABLE 5.9

Technic of Presumptive Test for Infectious Mononucleosis

Tubes	Saline	Serum	Serum Dilutions	2 Per Cent Sheep Cells	Final Dilutions of Serum
	ml.	*ml.*		*ml.*	
1	.4	.1	1:5	.1	1:7
2	.25	.25 of 1:5	1:10	.1	1:14
3	.25	.25 of 1:10	1:20	.1	1:28
4	.25	.25 of 1:20	1:40	.1	1:56
5	.25	.25 of 1:40	1:80	.1	1:112
6	.25	.25 of 1:80	1:160	.1	1:224
7	.25	.25 of 1:160	1:320	.1	1:448
8	.25	.25 of 1:320	1:640	.1	1:896
9	.25	.25 of 1:640	1:1,280	.1	1:1,792
10	.25	.25 of 1:1,280	1:2,560	.1	1:3,584
11	.25	.25 of 1:2,560	1:5,120	.1	1:7,168
12	.25	.25 of 1:5,120	1:10,240*	.1	1:14,336
Control 13	.25	—	—	.1	

* Discard .25 ml. from last tube.

TABLE 5.10

The Presumptive Test for Infectious Mononucleosis

Interpretation of Results

The finding of a titer of 224 or higher, in a person who did not receive an injection of horse serum or of horse immune serum in the recent past, and who presents a clinical picture and hematologic findings suggestive of infectious mononucleosis, indicates with a high degree of probability the presence of infectious mononucleosis.

Antisheep agglutinins in titers up to 56 may be found in normal persons and in patients with a variety of diseases. Occasionally, also titers of 112 may be found in such persons.

When titers of 224 or higher occur in patients with serum disease, or following injection of horse immune serum;

When the presumptive test shows titers of 224 or higher, but the characteristic clinical or hematologic findings of infectious mononucleosis are absent;

When the test shows titers of 112 or less, but the characteristic clinical or hematologic findings of infectious mononucleosis are present.

Then the differential test for infectious mononucleosis is indicated.

and complete removal with beef cells which is characteristic for infectious mononucleosis. In my experience this test has been positive only in infectious mononucleosis and negative in all other diseases. It is a qualitative test and the height of titer is not important. The test is as a rule positive when the patient presents symptoms of the disease or when the patient is first examined by the physician. There are cases in which

TABLE 5.11

The Differential Test

Principle

Antisheep agglutinins in *infectious mononucleosis* are NOT absorbed by Forssman antigen (guinea pig or horse kidney); they are NOT of the Forssman type.

Antisheep agglutinins in *serum disease* and in *normal persons* are readily absorbed by Forssman antigen; they are of the Forssman type.

Antisheep agglutinins in *infectious mononucleosis* are readily absorbed by beef cells.

Antisheep agglutinins in *conditions other than infectious mononucleosis* may or may not be absorbed by beef cells.

Materials	Preparation of Antigens
1. Test tubes (a) for absorption: 85 x 13 mm. (inside diameter) rounded bottom and no lips. (b) for agglutination: 75 x 10 mm. (inside diameter) 2. Blood serum (0.5 ml. will suffice) inactivated for 30 minutes minutes at 56°C. 3. 2 per cent suspensions of sheep erythrocytes 4. Capillary pipets 5. Physiologic salt solution	1. Guinea pig kidney Organs are stored frozen until needed, then thawed and washed with physiologic saline until washings are free of blood. They are now mashed into fine pulp and made into 20 per cent suspension in physiologic saline. The suspension is boiled one hour on water bath, and loss by evaporation is made up with distilled water. 2. Horse kidney A 2 per cent suspension in physiologic saline is prepared. Otherwise the procedure is the same as for guinea pig kidney. 3. Beef red cells They are washed 3 times with physiologic saline, and packed by centrifuging. One volume of packed cells is suspended in 4 volumes of saline and the suspension is boiled on water bath for one hour. Loss by evaporation is made up with distilled water. To all antigens, phenol is added to make a 0.5 per cent solution. Can be kept in icebox for many months.

the test is negative early in the disease and may remain negative as long as three weeks. Therefore, in cases of negative tests it is necessary to repeat the test if clinical and hematologic findings still suggest the disease in the third week from onset. The test remains positive for weeks and sometimes months after the disappearance of the clinical symptoms.

Details regarding the differential test are summarized in Tables 5.11, 5.12, 5.13, 5.14, 5.15.

TABLE 5.12

Differential Test

Procedure

1. Place in test tube (85 x 13 mm.) 1 ml. of thoroughly shaken Forssman antigen suspension.
2. Add 0.2 ml. of serum.
3. Shake, let stand at room temperature for 3 minutes.
4. Centrifuge at 1500 r.p.m. for 10 minutes or longer till supernatant is perfectly clear.
5. Remove the supernatant fluid with a capillary pipet, making sure not to take particles along.
6. Set up as many tubes (75 x 10 mm.) as needed, according to the titer of the presumptive test. Add 0.25 ml. of physiologic saline to each tube, except the first.
7. Add 0.25 ml. of the absorbed serum to the first and second tubes.
8. Mix the second tube and transfer 0.25 ml. to the third tube, etc. Discard 0.25 ml. from the last tube. The serum dilutions are 1:5, 1:10, 1:20, etc.
9. Add 0.1 ml. of the 2 per cent suspension of sheep cells. Shake well. Final dilutions of serum are 1:7, 1:14, etc.
10. Absorption with boiled beef cells. Follow the same procedure as above, using 1.0 ml. of the thoroughly mixed 20 per cent suspension of beef cells.
11. Let stand at room temperature. When speed is indicated reading may be done after 15 minutes. If test is positive, which means:
    (a) in test tubes containing serum treated with Forssman antigen, agglutination in the same dilution as the presumptive test or in not more than three dilutions or tubes below that of the titer of the presumptive test, and
    (b) no agglutination in test tubes containing serum treated with beef cell antigen,

    then the test can be reported as positive for infectious mononucleosis.

    If the result is negative, which means:

    more than 3 tubes difference in the agglutination as compared with the titer of the presumptive test (and/or agglutination remaining after absorption with beef cell antigen),

    repeat reading at intervals as frequent as convenient. Final negative results should NOT be recorded until after two hours incubation.

    If speed is not a factor, results may be read only once, at the end of two hours.

TABLE 5.13

The Differential Test

Interpretation of Results

In serum of patients with infectious mononucleosis, absorption with Forssman antigen will result in a partial removal of the agglutinin for sheep red cells, but, as a rule, not less than one-fourth of the original titer will still be present in the serum after absorption. For example, an original titer of 1:112 may be reduced to 1:28 after absorption with Forssman antigen.

If all or almost all (more than 90 per cent) of the sheep agglutinins have been removed by absorption with Forssman antigen, this speaks against infectious mononucleosis.

On the other hand, so far no other condition has been found, except infectious mononucleosis, in which Forssman antigen fails to remove sheep agglutinins from the serum.

The absorption with beef red cells is a confirmatory procedure in infectious mononucleosis, since absorption with beef cells removes all or almost all (more than 90 per cent) of the sheep agglutinins from the serum. Failure of beef cells to remove sheep agglutinins speaks against infectious mononucleosis.

It may be preferable to do the absorption with Forssman antigen and with beef cell antigen in EVERY case. The absorption with beef cells is essential in cases of patients with elevated titers of antisheep agglutinins if no clinical or hematologic findings are present suggestive of infectious mononucleosis. In most of these instances the antisheep agglutinins will be completely removed by Forssman antigen, but, in rare instances, the absorption may be incomplete. The use of the beef cell antigen will then be decisive.

Examples

	Titer of			
	Presumptive test	Differential test after absorption with		Result
		Forssman antigen	Beef red cells	
(a)	1:224	1:112	0	POSITIVE for infectious mononucleosis
	1:224	0	1:112	NEGATIVE for infectious mononucleosis
	1:224	1:56	0	POSITIVE for infectious mononucleosis
	1:224	1:56	1:56	NEGATIVE for infectious mononucleosis
	1:224	1:28	0	POSITIVE for infectious mononucleosis
	1:224	1:14	0	NEGATIVE for infectious mononucleosis
	1:224	1:7	0	NEGATIVE for infectious mononucleosis
(b)	1:56	1:56	0	POSITIVE for infectious mononucleosis
	1:56	1:56	1:28	NEGATIVE for infectious mononucleosis
	1:56	1:28	0	POSITIVE for infectious mononucleosis
	1:56	1:14	0	POSITIVE for infectious mononucleosis
	1:56	1:7	0	POSITIVE for infectious mononucleosis
(c)	1:28	1:28	0	POSITIVE for infectious mononucleosis
	1:28	1:14	0	POSITIVE for infectious mononucleosis
	1:28	1:7	0	POSITIVE for infectious mononucleosis

TABLE 5.14

Tests for Infectious Mononucleosis

Diagnosis	I. Presumptive Test	II. Differential Test			
		A		B	
	SRBC[a] agglutinated	Antibody absorbed by FA[b]	SRBC Agglutinated after absorption	Antibody absorbed by BCA[c]	SRBC agglutinated after absorption
(1) Forssman heterophilic antibody in normal serum	Yes (1:56 or lower in about 98%)	Yes	No	[d]	[d]
(2) Forssman heterophilic antibody in horse serum sickness or sensitization	Yes (usually 1:112 or higher)	Yes	No	Yes	No
(3) Various assorted conditions	Yes (may be elevated)	Yes or no	Yes or no	Yes or no	Yes or no
(4) Heterophilic antibody of infectious mononucleosis	Yes (as a rule elevated but may be 1:56 or less)	No[e]	Yes	Yes	No

[a] Sheep red blood cells.

[b] Forssman antigen.

[c] Beef cell antigen.

[d] Absorption is incomplete in approximately $\frac{1}{3}$ of normal persons; if absorption is incomplete, agglutination is present.

[e] Titer after absorption not more than 3 tubes lower than in presumptive test.

TABLE 5.15

Positive Differential Test in Cases with Low Titers in Presumptive Test
(1:56 *or less*)

	Number of tests	Tests with titers of 1:112 or less
First tests (early in disease)	267 (in 267 patients)	46 (17.2%)
Follow-up tests (3 to 18 weeks after onset of symptoms)	107 (in 69 patients)	39 (36.5%)
Total number of tests	374 (in 267 patients)	85* (22.7%)

* In these 85 tests infectious mononucleosis could *not* be diagnosed with the *presumptive* test.

Only the *differential* test made it possible to establish the *serologic* diagnosis of the disease.

The etiology of the disease is not known although it is suspected that it is due to a virus. Successful transmissions of the disease from monkey to man, from man to monkey, and from man to man have been reported but require confirmation.

MONOCYTOSIS (PLATE 5.I)

Definition: increase above the normal of 500 per cu. mm.

Monocytosis is present during the recovery stage from acute infections and is usually considered a favorable sign except in tuberculosis. An increase of monocytes in tuberculosis is a bad prognostic sign. An index has been developed expressing the ratio of monocytes to lymphocytes. The absolute number of lymphocytes is divided by the absolute number of monocytes; and if the quotient is higher than 1, the prognosis is better than if it is lower.

Monocytosis may be present in endocarditis lenta, due to streptococcus viridans. In this condition monocytes may show phagocytosis of other blood cells, red blood cells, leukocytes, etc. It may be present in Hodgkin's disease, as well as in various lipoid storage diseases, Gaucher's disease, Niemann-Pick's disease, etc.

BLOOD AND MARROW FINDINGS IN LESIONS AFFECTING BLOOD PRODUCING TISSUES: LYMPHOMAS

The terminology of lesions referred to in the heading of this chapter is still in a state of confusion. For example, some use the term lymphoma to indicate malignant lesions of lymphoid tissues. Some include all neoplastic or borderline neoplastic or hyperplastic lesions of blood producing tissues under this heading. One popular medical dictionary defines lymphoma as "a benign tumor composed of lymphatic tissue." Another dictionary defines it as "any tumor made up of lymphoid tissue."

HODGKIN'S DISEASE

Classification by Jackson and Parker

They recognize three forms: paragranuloma, granuloma and sarcoma. The paragranuloma is comparatively benign and the microscopic picture shows least variation from the normal, but each of them contains the Sternberg-Reed cells.

The paragranuloma shows no necrosis and the predominant cell is the adult lymphocyte. Sternberg-Reed cells are few. Eosinophils are rare. Plasma cells are quite common. It may pass into granuloma at any time.

TABLE 5.16

*Classification of Lymphomas**

General Classification	Subclassification	Terms to Be Avoided
Lymphoma		Lymphoblastoma
Type unclassified		
Lymphocytic sarcoma		Lymphosarcoma
Reticulum cell sarcoma		Reticulosarcoma Reticulo-endotheliosis
Follicular lymphoma		Giant follicular lymphoma Giant follicular lymphoblastoma Brill-Symmer disease
Hodgkin's disease	Hodgkin's paragranuloma Hodgkin's granuloma Hodgkin's sarcoma	Hodgkin's lymphoma Hodgkin's lymphoblastoma Hodgkin's lymphogranulomatosis

* Modified from Table 1 in Fourth Report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs. Am. J. Clin. Path. 20: 562–579 (June) 1950.

Many authors doubt that paragranuloma is an entity as distinctly discernible as claimed by Jackson and Parker, especially on histologic grounds. On the other hand the great variation in the clinical course is undeniable.

Blood Picture

Normocytic, sometimes severe anemia is seen in about 50 per cent of cases.

Leukocyte count may be elevated, normal or reduced (leukopenic). Differential count: neutrophilia, lymphocytopenia, monocytosis and eosinophilia. Either all or any combination of them may be present.

Neutrophilic leukocytosis, especially when lymph nodes involved. Neutropenia when bone marrow is involved. The blood changes seem to depend on the stage of the disease and on some individual as yet unknown factors.

The most frequent finding is a moderate leukocytosis with white cell

counts ranging from 12,000 to 25,000 per cu. mm. and a relative and even absolute lymphopenia. In the differential count the lymphocytes may go down below 10 per cent. The pathogenesis of this change is impairment of lymphocytogenic properties of reticulo-endothelial tissues by the disease. As a rule lymphopenia is prognostically a bad omen. Lymphocytic sarcoma may show lymphopenia similar to that seen in Hodgkin's disease except that as a rule there is no leukocytosis.

Monocytosis is frequent, with values of 10 per cent or higher. Eosinophilia was described in about 20 per cent of cases. It may be extreme. Platelet count may be increased, normal, or decreased, especially with marrow involvement.

Marrow Findings

Frequently there is a shift to the left of granulocytes, slight monocytosis, and eosinophilia. As a rule findings are not characteristic.

Amyloid degeneration of various grades is sometimes present in various organs, and with it plasma cell hyperplasia in the marrow.

Marrow may be diagnostic when Hodgkin's granuloma with its characteristic pattern including Sternberg-Reed giant cells is seen in a biopsy and occasionally even in marrow puncture smears.

LEUKEMIA

Definition

A disease of the white blood cells producing tissues characterized by systemic hyperplasia of leukocytes (granulocytes, lymphocytes or monocytes) and their precursors, frequently but not invariably with corresponding quantitative and qualitative changes in the circulating white blood cells.

There are at the present time three different conceptions regarding the nature of leukemia.

1. Leukemia is a hyperplastic condition of the marrow, lymphatic, or reticulo-endothelial tissues as the case may be.

2. It is a neoplasm of the corresponding tissues.

3. Leukemia is closely related to neoplastic diseases arising in hemopoietic tissues. According to this opinion the malignant nature of leukemias has not been definitely established.

Classification

According to the consensus of the members of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs, the leukemias should be classified in the Standard Nomenclature of

TABLE 5.17

*Classification of Leukemia**

General Classification	Subclassification	Terms to Be Avoided	Predominant Cell	Variants	Longevity
Leukemia Blast cell (type unclassified)	Acute[a] leukemic[b] subleukemic[c] aleukemic[d]	Stem cell leukemia	Unclassified blast cell (undifferentiated)		Under 12 mo.
Lymphocytic	Acute leukemic subleukemic aleukemic	Lymphoblastic leukemia Blast cell leukemia	Lymphoblast		Under 12 mo.
	Subacute[e] leukemic subleukemic aleukemic	Lymphadenosis Lymphatic, lymphoid or lymphogenous leukemia	Partially differentiated lymphocyte	Associated with lymphocytic sarcoma	12–24 mo.
	Chronic[f] leukemic subleukemic aleukemic		Well differentiated lymphocyte		Over 24 mo.
Granulocytic Neutrophilic	Acute leukemic subleukemic aleukemic	Myeloblastic leukemia	Myeloblast	Micromyeloblastic Monocytoid (Naegeli) Chloroma	Under 12 mo.
	Subacute leukemic subleukemic aleukemic	Myeloid, myelogenous, myelocytic leukemias	Partially differentiated granulocyte		12–24 mo.
	Chronic leukemic subleukemic aleukemic	Myelosis	Well differentiated granuloctye	Subclinical Eosinophilic Basophilic Leukoerythroblastic Polycythemic Thrombocythemic Megakaryocythemic Megakaryocytic Acute relapsing Myelofibrotic Osteosclerotic	Over 24 mo.

TABLE 5.17 —*Continued*

General Classification	Subclassification	Terms to Be Avoided	Predominant Cell	Variants	Longevity
Monocytic	Acute leukemic subleukemic aleukemic		Monoblast		Under 6 mo.
	Subacute leukemic subleukemic aleukemic	Reticulo-endotheliosis Schilling or Naegeli type leukemia	Partially differentiated monocyte	Associated with reticulum cell sarcoma	6–12 mo
	Chronic leukemic subleukemic aleukemic		Well differentiated monocyte		Over 12 mo.
Plasmocytic	Acute leukemic subleukemic aleukemic		Plasmoblast		
	Subacute leukemic subleukemic aleukemic		Partially differentiated plasmocyte	Associated with multiple myeloma of corresponding degree of cell maturity	Unpredictable
	Chronic leukemic subleukemic aleukemic		Well differentiated plasmocyte		

* Modified from Table 1 in Fourth Report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs. Am. J. Clin. Path. 20: 562–579 (June) 1950, and from Table 22 in R. Philip Custer: An Atlas of the Blood and Bone Marrow. Philadelphia: W. B. Saunders, 1949, p. 244.

[a] Acute leukemias have an expected duration of life which is counted in months from the onset of the first symptom. The onset is typically with fever, stomatitis and bleeding into the skin, from the gums and mucous membranes. The predominant cell in the marrow and in the blood in leukemic cases is the blast form, with the single exception that in some of the acute leukemic lymphocytic leukemias, it is a cell indistinguishable morphologically from the lymphocyte or prolymphocyte. Except in the lymphocytic type, cells more differentiated than the pro- stage of the involved series are rare or absent.

[b] Leukemic leukemias are defined as those leukemias in which the leukocyte count in the blood is above 15,000 per c.mm. and the type cells are present in the blood in sufficient number to permit the diagnosis of the type of leukemia.

[c] Subleukemic leukemias are defined as those leukemias in which the leukocyte count in the blood is below 15,000 per c.mm. It is often much below normal, in the 100 to 4000 range, yet the type cells of the particular variety of leukemia are present in the blood in sufficient numbers to suggest the diagnosis.

[d] Aleukemic leukemias are defined as those leukemias in which the leukocyte count in the blood is below 15,000 per c.mm., often as low as 100 to 4000 per c.mm., but the type cells either are absent or

Table 5.17—Concluded

are so few, usually less than one per 1000 cells, that it is impossible to make the diagnosis from examination of the blood alone.

[e] Subacute leukemias are defined as those leukemias with an expected duration of life longer than in acute, but shorter than in chronic leukemia, from the onset of the first symptom. The onset is more insidious than in acute cases, but all develop fever, stomatitis and bleeding into the skin, from the gums and mucous membranes within three months from the onset of symptoms. The predominant cell in the marrow and blood in leukemic cases is the pro-stage of the involved series, and an increased number of blast cells is always found. Except in the lymphocytic type, cells more differentiated than the pro-stage of the involved series are rare or absent.

[f] Chronic leukemias are defined as those leukemias with an expected duration of life of one to twenty years or more, average three to five years, depending on the type, from the onset of the first symptom. Fever, stomatitis and hemorrhages into the skin or from the gums and mucous membranes rarely develop within the first year. The most differentiated stage of the involved series is always present, in addition to the less differentiated cells. In the lymphocytic type only the lymphocyte is usually found and prolymphocytes are rare or absent.

Disease (Jordan) under the etiologic category of "new growth originating in specialized mesenchymatous tissue." Favoring the inclusion of leukemias under malignant tumors, in the judgment of the Committee, is the fact "that the therapy currently used for these diseases (i.e., leukemias) is the same as is used for unquestioned malignant neoplasms."

The 4th report of the Nomenclature Committee defines leukemias as neoplastic diseases arising in hemopoietic tissues in which the type cells appear in the blood or are disseminated diffusely through the marrow. If the cell series cannot be identified, then the leukemia should be included in the category "type unclassified." Usually that means an acute leukemia of unidentified "blast cell" type. For new nomenclature of blood cells see Chapter II, sections on granulocytes and lymphocytes.

According to the report of the Committee, the name of the most undifferentiated of the cells of each series of white blood cells should carry the suffix-blast, the second stage the prefix pro-, and except in the granulocytic series all cells that are more mature than the -blast stage have names with the suffix -cyte. The name of the fourth stage in the granulocytic and erythrocytic series is to have the prefix meta-. The terms -blast cells and pro- cells may be used to replace other terms for these stages of development when speaking of the stage of development as a whole or when the series to which the cells belong has not been identified.

Summary

I. Classification
   1. Chronologic, based on duration
      a. fulminant
      b. acute
      c. subacute
      d. chronic
   2. Cytologic, based on predominant cell
      a. blast cell leukemia, for undifferentiated types
      b. granulocytic leukemia
         acute (myeloblastic)
         chronic

c. lymphocytic leukemia
   acute (lymphoblastic)
   chronic
d. monocytic leukemia
   acute (monoblastic)
   chronic
e. neutrophilic leukemia
f. eosinophilic leukemia
g. basophilic leukemia
h. thrombocytic leukemia
i. plasmocytic leukemia
j. lymphocytic sarcoma cell leukemia

3. Based on functional capacity of release mechanism (marrow-blood and tissue-blood barrier)
   a. leukemic
   b. subleukemic
   c. aleukemic
4. Based on association with a definite neoplastic lesion of the same cytologic composition as the leukemic cells
   a. chloroma
   b. myeloblastoma
   c. myeloma (plasma cell leukemia)
   d. lymphocytic cell sarcoma

II. General data
1. Age
   a. all forms may occur at any age
   b. acute leukemia most frequent below 20
   c. chronic granulocytic most frequent between 20 and 50
   d. chronic lymphocytic most frequent over 50
2. Sex: males more commonly affected

III. Etiology—unknown except for isolated observations of etiologic significance
1. Eight to ten times as frequent in radiologists as in other medical specialists
2. Hereditary predisposition in man suggestive
3. Various agents including radiation, capable of producing the disease in animals and man
4. Transmissible in some animals by cell-free filtrate
5. Transmissibility in man not demonstrated

IV. Chronic leukemia
A. Clinic
1. Insidious onset; not infrequently symptomless for a long time, especially chronic lymphocytic leukemia, so-called subclinical leukemia
2. Significant clinical findings

	Granulocytic	*Lymphocytic*
Lymph nodes	Moderate enlargement, late	Early and marked
Spleen	Markedly enlarged	Moderate enlargement
Skin lesion		More common
G-I tract infiltration	Rare	More common

Bone tenderness	Rare	Moderately frequent (sternum)
Retinopathy	Common	Uncommon

3. Symptoms from
   a. enlarged spleen, liver, lymph nodes and other organs, are due to
      (aa) anatomic enlargement
      (ab) interference with specific function of organ by replacement of its normal tissues by leukemic infiltration
      (ac) interference with function of neighboring organs by pressure
      (ad) infarction of spleen with pain and tenderness
   b. Symptoms from anemia are due to cardiac dilatation, tachycardia, palpitation, dyspnea, cardiac failure, edema; anemia aggravates prognosis
   c. Symptoms from increased metabolic rate are due to weight loss, nervousness, perspiration, cachexia; this leads occasionally to diagnosis of hyperthyroidism
   d. Marrow replacement (usually late in disease) may result in hemorrhagic tendency from thrombocytopenia; aggravates prognosis
   e. Pain in bones, most frequent in chronic lymphocytic leukemia, but also in other forms; x-ray may show osteoporosis and periosteal infiltration

B. Laboratory findings
   1. Blood thick due to large number of leukocytes
   2. Heavy buffy coat on centrifugation of blood treated with anticoagulant
   3. Variable anemia, usually late. Occasionally high red counts
   4. Platelet count
      a. Frequently increased in granulocytic form with terminal drop
      b. May be decreased late in lymphocytic form
      c. Low counts are of serious prognosis
      d. Hemorrhage may occur with normal and high platelet counts. Cause: vascular damage
   5. Marked leukocytosis
      a. More frequent in granulocytic leukemia (100,000–800,000)
         Segmented forms 30–70 per cent
         Myelocytes 30–50 per cent
         Blast cells 0–10 per cent
         Both eosinophilic and basophilic forms are increased
      b. Lymphocytic form has monotonous picture with 90–99 per cent adult small lymphocytes. Total count about 100,000 with lower and moderately higher figures common
      c. Normal counts and leukopenic forms are not unusual
   6. Urine: albumin, casts, RBC
   7. Basal metabolic rate elevated in chronic granulocytic and late in chronic lymphocytic leukemia
   8. Blood chemistry: elevated uric acid and total non-protein nitrogen. Sometimes negative nitrogen balance. Low plasma protein (in advanced cases). Lipids may be elevated. Cholesterol normal or low. Phosphorus and serum alkaline phosphatase may be elevated

V. Acute leukemia (Plate 5.II)
  A. Clinic
    1. Onset sudden and course short
    2. Weakness, prostration, rapidly developing anemia, hemorrhagic diathesis, and gingivitis
    3. Joint and periarticular pain are not infrequent with bone involvement
    4. Disease is fulminating in nature and may resemble a sepsis
    5. Organ enlargement and infiltration are usually less conspicuous
    6. Features are found in the following types most frequently but not enough for differential diagnosis
      a. Monocytic
        (aa) Ulcerative gingivitis
        (ab) Cervical gland enlargement and occasional tenderness
      b. Lymphoblastic
        (ba) Splenomegaly
        (bb) Liver enlargement (50 per cent)
        (bc) Generalized lymphadenopathy
    7. Marrow studies are indicated for differentiation of the forms
  B. Laboratory findings
    1. Data applicable to all acute leukemias
      a. At first moderate, later rapidly developing severe anemia, normochromic and normocytic. Nucleated red cells present
      b. Low platelet count (if normal, question diagnosis). Rise of platelet count may be earliest sign or remission
      c. Prolonged bleeding and clot retraction time
      d. Occasionally prolonged clotting time
      e. Hemorrhagic tendency (positive tourniquet test)
      f. Hemocytology: count elevated (moderately up to 100,000, frequently much lower); may be normal, or even subnormal (leukopenic). Type cell: blast cell of one of three types. Few or no intermediate cells, up to level of segmented cells, lymphocytes or monocytes (leukemic hiatus)
      g. High basal metabolic rate
      h. Elevated uric acid and total non-protein nitrogen. Nitrogen balance may be negative. These changes may be present also in chronic leukemia, but less frequently
      i. Marrow: replacement with blast cells. Undifferentiated in blast cell leukemia and correspondingly differentiated in the other three forms of acute leukemia
    2. Acute granulocytic
      a. Type cell: myeloblast. Small numbers of progranulocytes. No further intermediate cells till level of segmented cell, leukemic hiatus. Sometimes micromyeloblasts may be difficult to differentiate from lymphocytes
      b. Auer bodies (rod-shaped cytoplasmic inclusions) if present exclude acute lymphocytic leukemia
    3. Acute lymphoblastic
      a. Type cell: lymphoblasts. Occasional prolymphocytes; remainder lymphocytes

4. Monocytic (Plate 5.II)
   a. Type cell: monoblast; occasional promonocytes; remainder monocytes. Auer bodies exclude acute lymphocytic leukemia
   b. Naegeli type. Acute granulocytic leukemia with transient monocytic phase
   c. Schilling type. True monocytic leukemia
5. Blast cell leukemia if differentiation of blast cells impossible. Presence of even small numbers of progranulocytes, prolymphocytes and promonocytes helps to identify type of acute leukemia

Discussion

The volume of writing on the cause and nature of the disease is inversely proportional to what is actually known about it. The fatal outcome is one of the few things on which there is fairly general agreement, but there are dissenting voices even on this point.

Leukemia is a collective term which includes several entities. The nature of the disease is largely a mystery. Isolated bits of information have been accumulating which will eventually add up and explain the puzzle.

Several factors have been discovered which have some bearing on leukemia. Constitutional factor: familial inherited disposition in certain families with several members of the family affected. External factors: x-ray, benzol.

Neoplastic Nature of Leukemia. The neoplastic nature of the disease in animals has been established by experimental studies. The same transplant may produce in some animals leukemia, in others true tumors. Intravenous injection of leukemia cells, even of a single cell, produces leukemia. Carcinogens, such as benzpyrene, methylcholanthrene and x-rays, have produced experimental leukemia.

Tumor-like growth and transitions between tumors and leukemia have also been observed in animals as well as in man.

Arguments in favor: Tendency to grow without restraint. Shift in ratio of nucleus to cytoplasm in favor of nucleus. Increased number and size of nucleoli. Atypical mitoses.

Arguments against: So-called primary tumor in the usual form cannot be demonstrated. The disease has a pronounced systemic character and distribution.

The leukemic lesions in various tissues and organs have been interpreted as metastases by some authors who favor the neoplastic hypothesis. The argument against such interpretation runs as follows: there are cases of extramedullary myeloid metaplasia in septic infections, in anemias and in osteosclerosis, which certainly have nothing to do with

tumors, and in which the extramedullary myelopoiesis can in no way be differentiated from similar lesions in a true granulocytic leukemia. The same holds true for material obtained by sternal puncture in cases of granulocytic hyperplasia, which frequently cannot be differentiated from chronic granulocytic leukemia.

Another argument against the neoplastic hypothesis is the multiformity of cellular structure. All stages of development of granulocytic and erythroid cells, including megakaryocytes, are represented. Such degree of differentiation speaks against neoplastic nature. It is true that in acute granulocytic leukemia the lesion is monotonously myeloblastic. Hence the inference by some that at least this form of leukemia is a neoplasm. Even that cannot be sustained because in some cases of agranulocytosis one finds in the marrow nothing but myeloblasts, a reversible lesion which as a rule returns to normal.

It is on the basis of all these observations that some have concluded that leukemias are hyperplastic systemic diseases.

The fact that leukemia is irreversible and as a rule fatal is used by some in support of its neoplastic nature. The answer to this is that prior to the discovery of liver therapy pernicious anemia had had the same attributes. The advocates of the hypothesis of neoplasia argue that in some cases of leukemia there are localized tumor-like proliferations with malignant type of growth, and that there are true tumors of blood-producing organs which show transitions to leukemias. However, these are isolated cases with features which set them apart from the usual case of leukemia. They are not sufficiently convincing to be used as a basis for developing a hypothesis regarding the nature of all leukemias. It is possible that leukemia may be associated with a true neoplasm. There is a difference between recognizing leukemia as a tumor and the possibility that a tumor may develop on the basis of a leukemia.

According to some authors leukemia is a precancerous stage of a cancer of hematopoietic tissues. The experiments in animals could then be explained in such a way that in cases where leukemia has already become transformed into cancer, a transplantation of cells at that stage of leukemia would produce cancer.

Nature of Acute Leukemia. According to some authors acute leukemia is a form of leukemia, equivalent to other forms. According to others it is not a true leukemia but a reactive process in response to certain stimuli (for example, septic infections). On the other hand, it is possible that the infections are the result and not the cause of acute

eukemia. Also other stimuli are considered as possible causes of acute eukemia, such as benzol, x-rays, and various drugs (for example, sul-'onamides).

Much has been written about cases of cured acute leukemias. As a 'ule, such reports must be viewed with great suspicion. However, when 'ases of this kind have been observed by reliable hematologists, includ-ng Naegeli, the possibility of their occurrence cannot be denied. Some 'ry to get around it by speaking of so-called "myeloblastic reaction." Some go even further and see in every acute granulocytic leukemia a 'eactive disturbance of the bone marrow and claim that the term acute eukemia does not apply to them.

There are true cancers of blood-producing organs which show hema-'ologic findings identical with those seen in certain forms of leukemia: 'hloroma, myeloblastoma, lymphosarcoma.

General Data on Leukemia

Incidence. In 1939–40, 5,286 deaths were attributed to leukemia in the United States, 0.39 per cent of all deaths and 3.6 per cent of deaths due to cancer. In the morgue population the incidence of leukemia has 'anged from 0.62 to 0.86 per cent in over 53,000 autopsies. It is worth noting that in 1950, 8,845 people died of leukemia, a death rate of 59 per 100,000 population (Kaplan). Chronic granulocytic leukemia is the most frequent form. Statistics on the relative incidence of various types of leukemia vary greatly but Rosenthal's statement comes pretty close to actuality. He claims that the relative incidence of granulocytes, lymphocytes and monocytes in the peripheral blood is about the same as the incidence of the corresponding three types of leukemia.

Occupation. More prevalent among physicians than among white males in the general population (1.7 times as frequent) and particularly frequent in radiologists and persons exposed to radiation. Among radiologists it is from 8 to 10 times as frequent as in non-radiologists. Chronic exposure to radiation is the probable explanation of the greater frequency among radiologists (Henshaw). This is supported by animal experiments. It seems to be also more frequent in restaurant workers and typographers (Gram).

Heredity. About 35 cases of multiple occurrence have been described in members of the same family. It may be important that in some instances of multiple occurrence different members of the families had different forms of leukemia.

Age. Up to age of 20 acute leukemia predominates, from 20 to 50

chronic granulocytic, above 50 chronic lymphocytic. Monocytic leukemia, which is most commonly acute, favors middle age and is rarely seen before the age of 30.

Sex. More prevalent in males, especially lymphocytic and monocytic.

Serologic Aspects. Patients with chronic leukemia are poor antibody producers. They have low titers of natural antibodies (antisheep agglutinins, isoagglutinins, etc.) (Davidsohn, d). Also immune antibodies may be low—for example, agglutinins for *S. typhosa* (Widal test) have been low in leukemic patients who contracted typhoid fever. This suggests that the reticulo-endothelial system of patients with chronic leukemia is inadequate. In acute leukemia the titers have been normal or high normal.

Experimental Production. In man, leukemia has been attributed to exposure to various chemicals (for example, benzol, pyridine, aniline dyes) and to x-rays. In animals, especially white mice, injections of benzol produce leukemia or leukemia-like conditions in about 15 per cent. Polycyclic hydrocarbons, estrogens and irradiation also produce leukemia. Even oral administration of methylcholanthrene produced leukemia in rats (Shay).

Leukemia occurs in several animal species and can be transmitted from animal to animal. The experimental conditions vary from species to species. In mammals viable cells must be used and there must be a close genetic relationship between the animals serving as donors and recipients. Cell-free filtrate or fragments of ruptured cells cannot transmit the disease. Carcinogens, x-rays and estrogens can induce leukemia in mice. Incidence of spontaneous leukemia in mice is decreased by thymectomy. Also diet low in cystine reduces incidence of leukemia induced by carcinogen. Spontaneous leukemia in mice is delayed by underfeeding. Immunity to transplanted leukemia can be produced by inoculation of sublethal doses of cells, injections of suspensions of normal tissue cells of certain genetic constitution, administration of heat-killed leukemic cells, of leukemic cell extracts, or of normal defibrinated blood.

Fowl leukemia can be transmitted by filtrate, therefore it is thought to be caused by virus. It has certain features of a neoplasm.

In man leukemia is not transmissible, not even by blood transfusions.

Acute Leukemia

General. The onset of the disease is sudden, and differs strikingly from the insidious onset of chronic leukemia. The disease resembles an acute infectious or even septic condition. Other changes include fever,

apidly developing anemia, signs of granulocytic insufficiency with ul-erations of mucous membranes, especially of mouth and throat, and purpura. Enlargement of lymph nodes, spleen and liver is not very pronounced. Rheumatoid pains, sometimes resembling acute rheumatic ever, are frequent. Marked prostration and general malaise may be present. The course is rapidly progressive. Acute leukemia may imi-ate a variety of diseases.

Some authors speak of fulminant leukemia when the course is ex-remely rapid and the patient dies in a few weeks.

Lymph node enlargement is conspicuous only in acute monocytic eukemia, especially cervical. Moderate generalized lymph node en-argement is more frequent in acute lymphocytic leukemia. In mono-cytic leukemia cervical lymph nodes are also tender.

The spleen is only slightly enlarged, except in acute lymphocytic leu-kemia when it may be very large.

Lesions in mouth, with swelling, ulceration and hemorrhages in the gums, are especially frequent in monocytic leukemia, but may also occur n other forms. Mucosal ulcerations may also be present in other parts of the gastro-intestinal tract. These lesions are again more frequent in nonocytic leukemia.

In addition to rheumatoid pains previously referred to there may lso be tenderness of bones and swelling and tenderness of joints.

The responsible pathologic lesion: subperiosteal leukemic infiltrations.

Blood Findings (Plate 5.II). Anemia is present almost without excep-ion—certainly in any case with fully developed clinical manifestations. t is usually normocytic. Frequently young nucleated red cells are present. Thrombocytopenia of moderate to marked degree is the rule with abnormally formed and excessively large platelets. Prolonged bleeding time and clot retraction time and positive tourniquet test are present. Coagulation time may be prolonged. Leukocyte count as a ule is slightly elevated, but may be normal or below normal. A com-bination of anemia, thrombocytopenia and leukopenia is a frequent inding in acute leukemia.

The frequent hemorrhagic tendency is explained by: (1) thrombo-ytopenia; (2) vascular damage, sometimes due to endothelial injury, nd sometimes to perivascular infiltration.

The predominant cell is an immature blast cell. Most frequently a myeloblast in the adult, a lymphoblast in the child. If differentiation s not possible, the term of blast cell leukemia may be used.

Acute Granulocytic Leukemia. *Cell type*: myeloblast.

Cell morphology. The nucleus shows a fine reticulated structure of chromatin, without condensations. There is no nuclear membrane. There are several distinct nucleoli. The cytoplasm is homogeneous without granules or with occasional azurophilic granules. More mature forms between the blast forms and mature granulocytes are absent or scarce. This gap between the immature myeloblasts and the mature segmented leukocytes is known as leukemic hiatus or gap and is characteristic for many cases of acute granulocytic leukemia

Presence of more than an occasional myelocyte and metamyelocyte suggests an acute phase or relapse of a chronic leukemia. The same holds if myeloblastic blood picture is present but thrombocytopenia and purpura are absent or if there is splenomegaly.

Auer bodies are characteristic: rod-shaped peroxidase positive bodies in cytoplasm of myeloblasts. They may be present occasionally in myelocytes or monocytes.

Acute Lymphocytic Leukemia. *Cell type:* lymphoblast.

Cell morphology. Granular or stippled chromatin, somewhat coarser than in a myeloblast. Usually only one or two somewhat indistinct nucleoli, not as many and not as sharply outlined as in a myeloblast. Chromatin tends to be condensed at the edges suggesting a nuclear membrane. There is frequently a clear perinuclear zone. Cytoplasm is basophilic, homogeneous, without granules. It is important to look carefully for granules in cytoplasm. If these are present, the possibility is that one deals with myeloblastic leukemia.

Some writers question the existence of an acute lymphocytic leukemia outside of early childhood.

Acute Monocytic Leukemia. *Cell type:* monoblast.

Cell morphology. Nucleus has a very delicate reticular chromatin in the form of a fine meshed network. The nucleus is frequently folded, indented, or coarsley and incompletely segmented. Nucleoli are less frequent and less prominent than in lymphoblasts, and still less than in myeloblasts.

Cytoplasm is basophilic with few or no granules. Outline of cytoplasmic margin is irregular and pseudopodium-like.

There is usually a good admixture of monocytes in various stages of maturation. In the monocytes the cytoplasm is greyish-blue, of ground glass appearance, with innumerable purplish-pink granules which are difficult to separate from each other.

Two forms of the disease are recognized. The first is the so-called Naegeli type which is a monocytic form of myeloblastic leukemia with

numerous myeloblasts; the number of monoblasts and monocytes varies from day to day and frequently gives way to an increase of myeloblasts. The second is the so-called Schilling type, which is a true monocytic leukemia. According to Downey, in the Schilling type the monocytes are derived from the reticuloendothelial system, and in the Naegeli type from myeloblasts.

Chronic Leukemia

Clinic. Disease may be present for a long time, even with increased white blood count and enlarged spleen and lymph nodes, before there are clinical manifestations of disease. The earliest signs of disease come from one or more of the following changes: pressure of enlarged spleen, liver, or lymph nodes. There may also be symptoms from increased metabolism, loss of weight, nervousness, perspiration, and deterioration of state of nutrition. Fever and bleeding occur late.

Lymph nodes are enlarged mainly in lymphocytic, less frequently in granulocytic leukemia. In either case enlargement is only moderate, and there are no adhesions to each other or skin. There is no tenderness, and no change in the skin.

In the spleen, enlargement is most pronounced in chronic granulocytic leukemia. Infarcts and perisplenitis may produce clinical symptoms.

Blood in Chronic Leukemia. *Macroscopic Appearance.* In advanced cases blood is thick and sticky, and even smears are difficult to prepare. In the hematocrit tube there is a thick layer of the buffy coat. This is particularly true in chronic granulocytic leukemia in which there is also a thick layer of platelets.

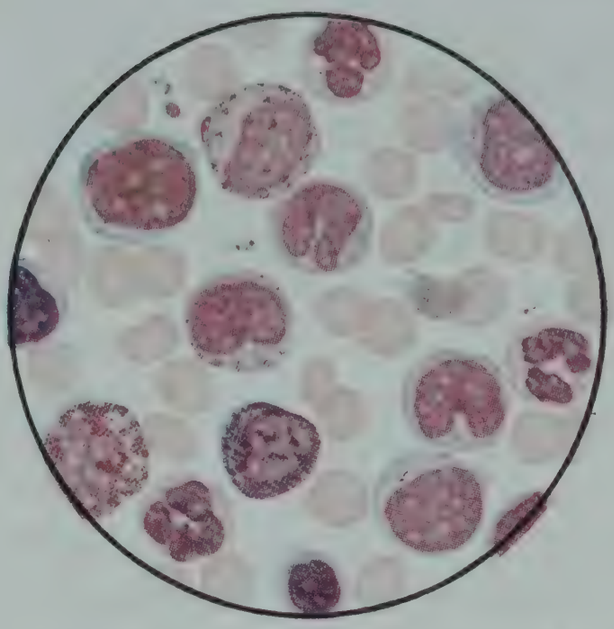
Red Cells. In early stages of chronic leukemia there may be no anemia but it appears sooner or later. Its intensity varies. As a rule, it is normocytic with anisocytosis; poikilocytosis is rare. Normoblasts, polychromatophilia, basophilic stippling and reticulocytosis may be present, especially in granulocytic leukemia, even with slight or moderate anemia, but not so in lymphocytic leukemia.

Platelets. Early in the disease the platelet count is normal or slightly increased. Later it may be markedly increased. Increase from subnormal to normal, or a fall from excessively high to normal is indicative of clinical improvement. Persistently low count is a bad sign.

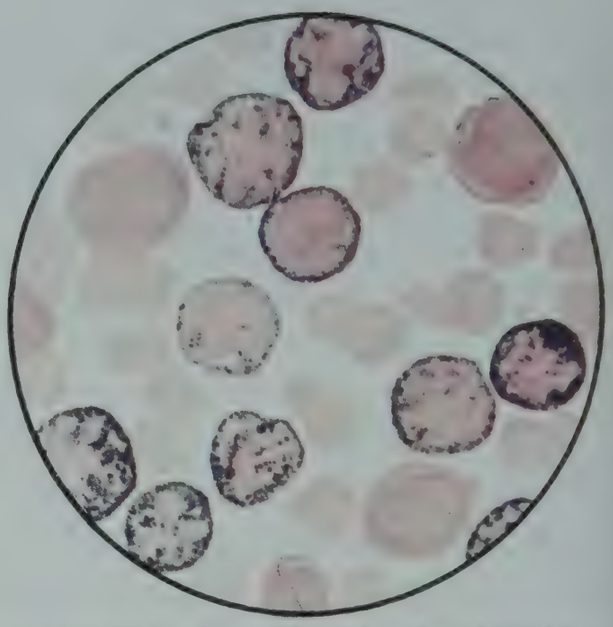
In chronic lymphocytic leukemia, platelet count is usually not increased, sometimes low and if considerably reduced the prognosis is bad.

White Cells (Plate 5.II). In chronic granulocytic leukemia the white cell count is markedly increased, ranging from 50,000 to 300,000 and

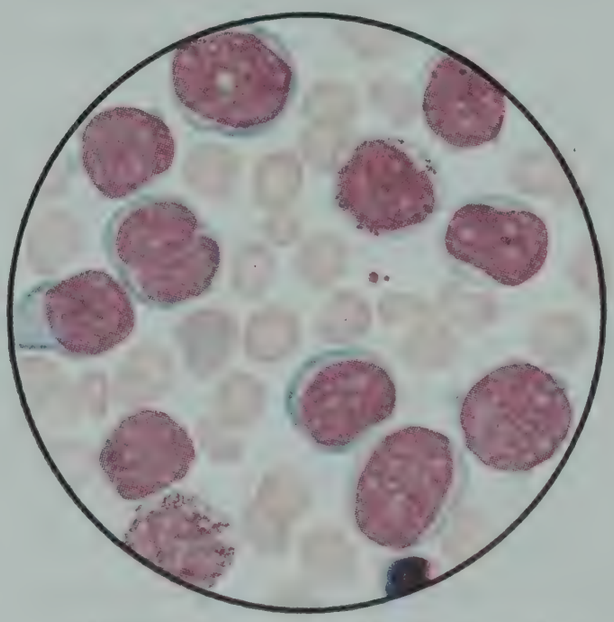
PLATE 5.II
THE LEUKEMIC STATES



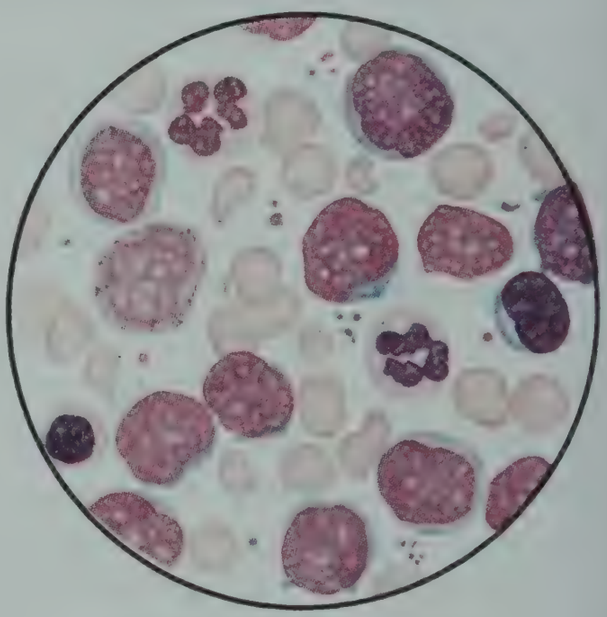
Chronic Granulocytic Leukemia



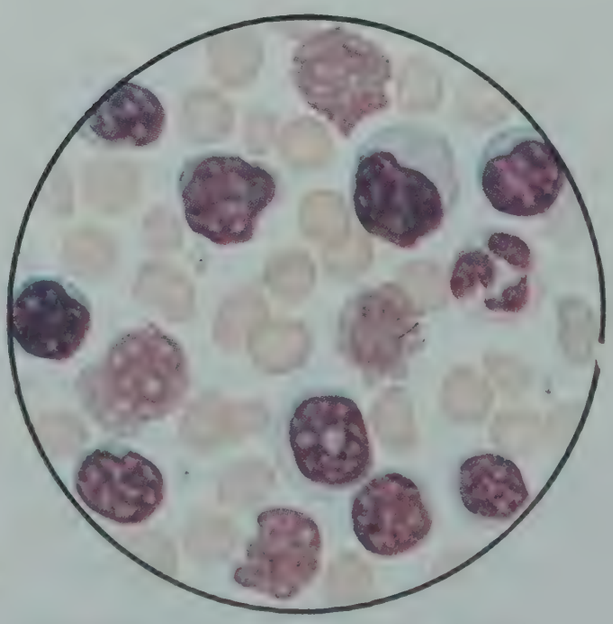
Chronic Granulocytic Leukemia
(Peroxidase Stain)



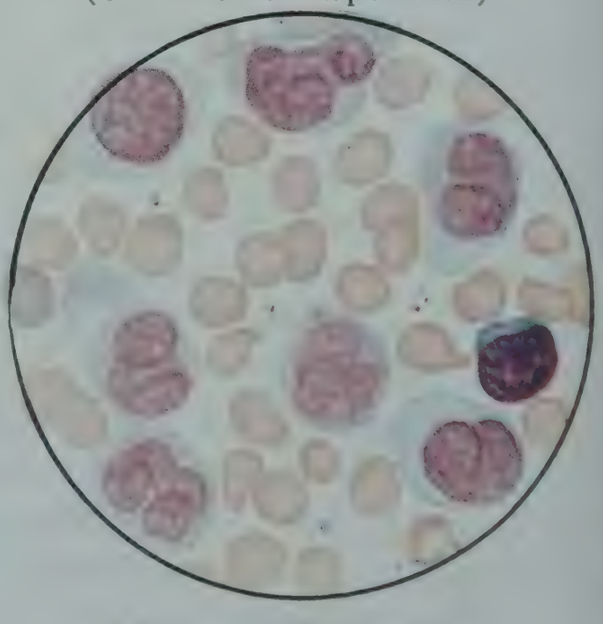
Acute Granulocytic Leukemia



Acute Granulocytic
Aleukemic Leukemia
(Concentrated Specimen)



Chronic Lymphocytic Leukemia



Monocytic Leukemia

even higher. Myeloblasts are usually few, myelocytes and metamyelocytes numerous. Myelocytes may be up to 50 per cent. Blood smear may be as cellular as a smear of a normal sternal marrow.

There is an increase of eosinophils and basophils. The latter may be from 3 to 20 per cent. The number of basophils is usually higher than in any other disease.

In chronic lymphocytic leukemia the white count is lower than in granulocytic, and ranges from 30,000 to 100,000. The differential blood picture is monotonous. It shows mainly small lymphocytes with very little cytoplasm. The number of cells with azurophilic granules and the number of azurophilic granules in the individual cells are reduced.

PLATE 5.II. The leukemic states

(Upper left): Chronic granulocytic leukemia. Leukocyte count was 120,000. The field contains 15 nucleated cells. At the extreme left is a small lymphocyte, at the extreme right a segmented neutrophil, at the extreme bottom a metarubricyte (normoblast), at the top a part of a segmented neutrophil. At the upper left is a myeloblast showing 4 nucleoli in its nucleus. At the lower right is a myeloblast showing 4 nucleoli with 2 Auer bodies in the cytoplasm. At the upper right is a premyelocyte with a few cytoplasmic granulations and 3 nucleoli in nucleus. At the lower left at 8 o'clock is a neutrophilic myelocyte and to its right is a basophilic myelocyte. The field also shows at the top a neutrophilic myelocyte and near the center 2 juveniles and 1 band.

(Upper right): A drawing of the same field as it would appear with a peroxidase stain. Note that the myeloblasts are peroxidase negative and the myelocytes positive. The lymphocyte and metarubricyte (normoblast) are peroxidase negative. All other cells give positive reaction.

(Center left): Acute granulocytic leukemia. The leukocyte count was 85,000. All cells shown are different types of myeloblasts with the exception that both cells showing cytoplasm granulation are myelocytes; 1 nucleated red cell at the lower border of the field. Note that the myeloblasts vary in size. Most of them show from 2 to 5 nucleoli in the nucleus. The cell at center left shows an atypical nuclear division. The cell at the upper left shows vacuoles in cytoplasm and nucleus. Note the irregular cytoplasm protrusions on some cells. At 8 o'clock the myeloblast appears to have no cytoplasm and appears to be all nucleus. Red cells show variations in size, shape and staining qualities. Note scarcity of blood platelets.

(Center right): Aleukemic acute granulocytic leukemia from a concentrated specimen of blood. The leukocyte count was 2,200, myeloblasts 65 per cent. The field shows 2 segmented neutrophils and 1 lymphocyte at the right, and 1 metarubricyte (normoblast) and 1 neutrophilic myelocyte at the left. The remainder of the cells are various types of myeloblasts. The leukocytes have been concentrated to facilitate study of the cells. Note numerous platelets because of concentration.

(Lower left): Chronic lymphocytic leukemia. The leukocyte count was 90,000. The field shows 1 segmented neutrophil, 9 lymphocytes, 3 smudge cells. Note that the predominating cell is the small lymphocyte.

(Lower right): Monocytic leukemia from a case of chronic monocytic leukemia. The leukocyte count was 44,000. Monocytes 86 per cent. The field contains 1 small lymphocyte at the lower right and 7 monocytes. Note that some of the monocytes show a fine dusty, reddish cytoplasmic granulation while others do not. The cytoplasm is light blue and is characterized by irregularity and protrusions. The nuclei are characterized by irregularity in size, shape and overfolding.

The nuclear chromatin is less dense than in normal lymphocytes. The cells vary only moderately in size. The nuclei, especially of the large lymphocytes, frequently show indentation.

Anemia in Leukemia. Causes of: 1) replacement of erythropoietic tissue in the marrow; 2) excessive hemolysis of red cells. Hemosiderosis is the anatomical evidence of the hemolytic process.

Anemia is frequently more severe in lymphocytic leukemia than in granulocytic, probably due to marrow replacement. Hypoplastic anemia (myelophthisis) may develop occasionally.

Increased basal metabolic rate is frequent in chronic granulocytic and lymphocytic leukemias and in acute leukemia, usually directly proportional to the severity of the disease. Cause: possibly, increased protein catabolism. After irradiation it rises for a few days then returns to normal for varying periods of time. Clinically there is a striking difference from the changes seen in exophthalmic goiter, although there is some nervousness, tremor and irritability. For example, there is frequently no loss of weight as in hyperthyroidism.

Microscopic tissue changes in chronic leukemia. *Granulocytic Leukemia.* Infiltrations of granulocytic cells in various organs, but usually earliest and most pronounced in spleen, liver and lymph nodes. They may be present in any and all tissues. The infiltrating granulocytic cells are of all grades of maturity. In the liver the infiltrations are, as a rule, more pronounced within the sinusoids in contrast to lymphocytic leukemia where they are located predominantly in periportal areas.

There are three schools of thought regarding the origin of the extramedullary myelopoietic foci in leukemia:

1. They are produced by metaplasia of multipotential mesenchyme
   a. in order to compensate for the marrow replaced or damaged by the leukemic process (compensation for marrow function)
   b. in response to the specific leukemic stimulus (enhancement of marrow function)
2. They are implants of the leukemic blood cells (colonization)
3. They are metastases from leukemic marrow.

In some instances the extramedullary foci are not limited to granulopoiesis but contain all three elements of marrow in varying proportions and are then referred to as foci of hematopoiesis.

Extramedullary hematopoiesis is not limited to leukemia. It occurs in any condition in which marrow is encroached upon by: 1) invasion of non-marrow tissue, for example, metastatic carcinoma; 2) by excessive growth of tissue normally present in the marrow (for example, hyper-

plasia of erythroid elements in pernicious or hemolytic anemia); 3) by hyperplasia of connective or osseous tissues, myelofibrosis or myelosclerosis, etc. Myeloid metaplasia in the spleen may assume tremendous proportions (so-called myeloid metaplasia syndrome).

Rohr claims that extramedullary granulopoiesis is the source of myeloblasts and myelocytes in the peripheral blood. This alone is apparently not sufficient as evidenced by aleukemic leukemias with splenomegalies. Apparently a release mechanism must be added to extramedullary myelopoiesis.

Lymphocytic Leukemia. The accumulations in various organs and tissues consist of lymphocytes. They are thought to be due to metaplasia of the mesenchyme. This explanation holds also for lymphocytic infiltrations in the marrow.

Marrow Findings (Plates 5.III, 5.IV)

Acute Leukemia. Diffuse and progressive replacement of marrow and fat by undifferentiated cells with thinning of trabeculae and cortex.

New chemotherapeutic agents (for example, aminopterin) have been reported to cause replacement of leukemic infiltrations by normal marrow.

Chronic Granulocytic Leukemia. *Gross:* grey or greyish-red, abundant, even in locations normally devoid of cellular marrow, for example, in long bones.

Microscopic: granulocytic hyperplasia. Change of E:G (erythrogranulocytic) ratio from the highest normal of 1:6 to about 1:10.

Cytology: increase of granulocytic cells of all types, with an overabundance of cells at each end of the granulocytic spectrum, myeloblasts and progranulocytes at one end and band and segmented forms at the other, and depression of nucleated red cells late in the disease. Frequently there is an increase of megakaryocytes, usually with an accompanying thrombocythemia. Eosinophilic and basophilic granulocytes are frequently more numerous.

Acute Granulocytic Leukemia. Many myeloblasts and undifferentiated red cells.

Eosinophilic Leukemia. Hyperplasia of eosinophilic granulocytes. Basophilic granulocytes may also be more numerous if the peripheral blood shows an increase

Lymphocytic Leukemia. Frequently no changes. In advanced cases there is replacement by lymphocytes. An increase of lymphocytes over 50 per cent is indicative of lymphocytic leukemia.

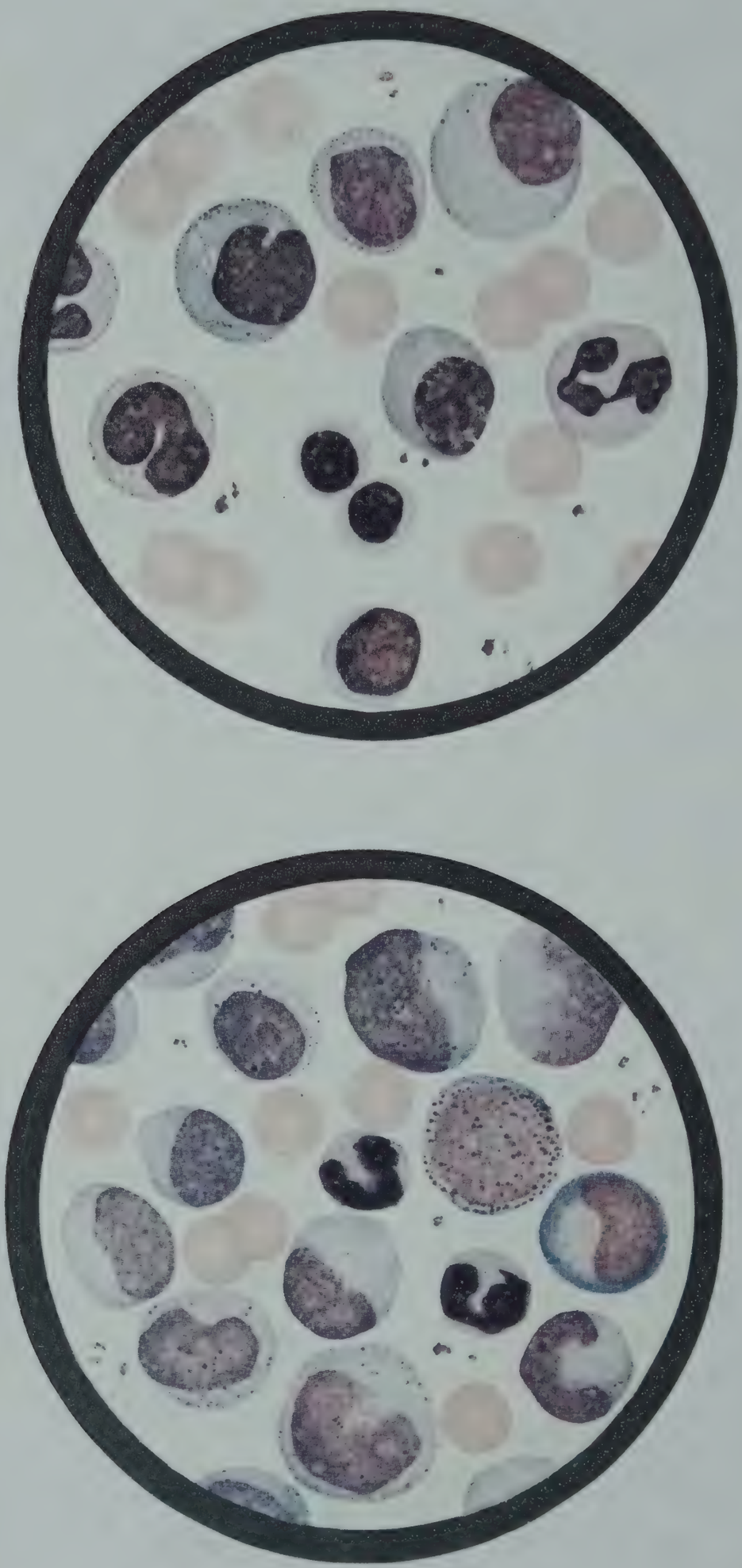


PLATE 5.III. Bone marrow aspiration smears.

Upper: Normal. Cells shown are lymphocyte (lower), metarubricytes (normoblasts) (lower center), segmented neutrophilic granulocyte (right), band cell (left), myelocytes (upper center), monocyte (upper right).

Lower: Chronic granulocytic leukemia. Cells shown are progranulocyte (lower), band cell (center), segmented neutrophile (right) and the remaining are early or late myelocytes, some showing nuclear degeneration.

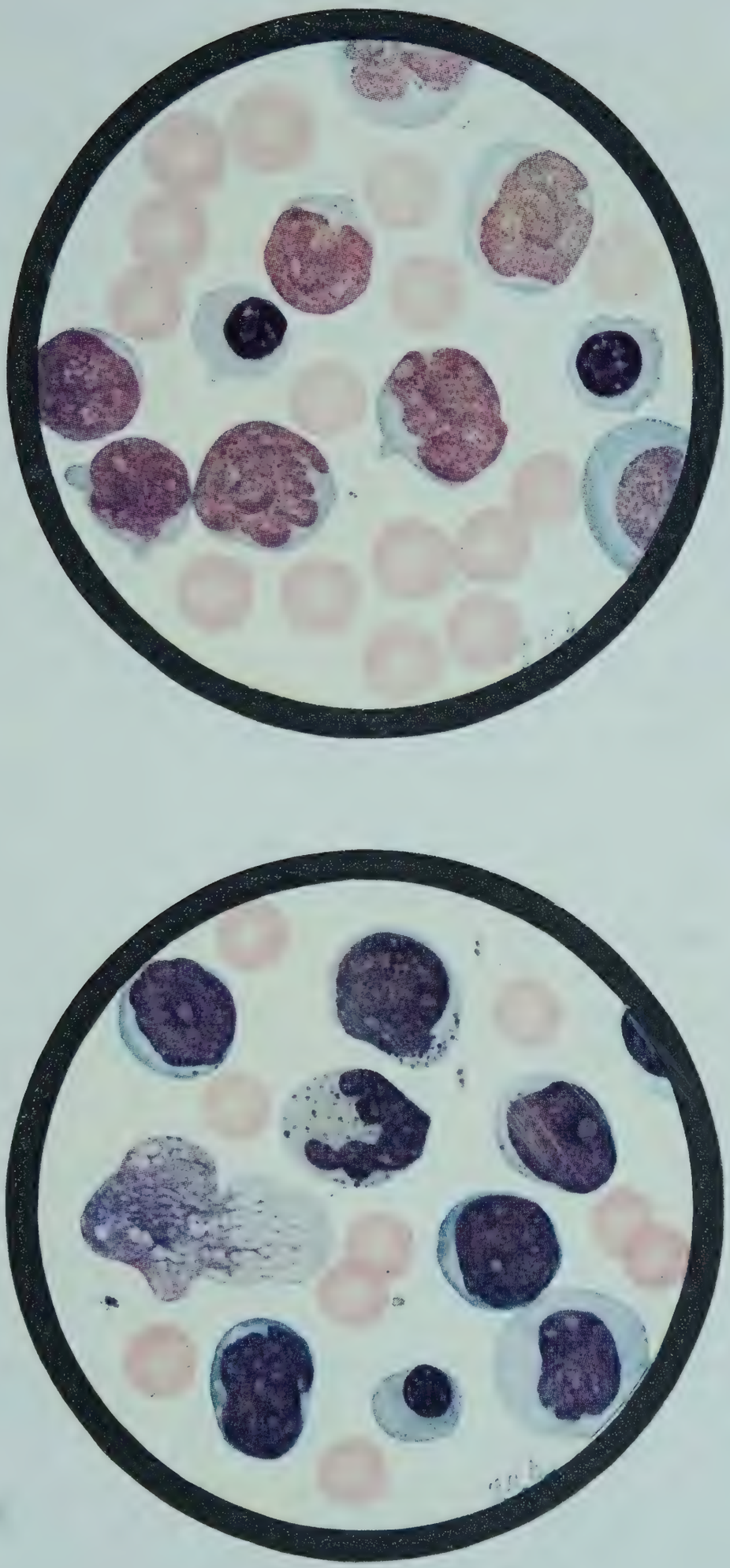


PLATE 5.IV. Bone marrow aspiration smears.

Upper: Acute granulocytic leukemia. Cells shown are myeloblasts with the exception of two metarubricytes (normoblasts) (center left and right), and a progranulocyte (right).

Lower: Acute lymphocytic leukemia. Six blasts are shown. The remaining cells are a disintegrating cell (left), segmented (center), and metarubricyte (normoblast) (lower).

Sources of error:

1. Admixture of peripheral blood; therefore, aspiration may be misleading;
2. Lymph follicles, which are present in about 10 per cent of older persons. Marrow biopsy may be more helpful.

Monocytic Leukemia. Large numbers of monoblasts, promonocytes and monocytes. Sections of marrow biopsies resemble a reticulum cell sarcoma.

Special Forms of Leukemia. *Plasmocytic Leukemia.* Almost always a part of multiple myeloma with corresponding marrow findings. Infiltration of various tissues with plasmocytes is present.

Thrombocytic (Megakaryocytic) Leukemia. Extremely rare and a doubtful entity. Most reported cases have probably been chronic granulocytic leukemia with excessive proliferation of megakaryocytes. Marrow shows hyperplasia of megakaryocytes of various grades of maturation, without accompanying granulocytic hyperplasia.

Chloroma. An acute myeloblastic leukemia with formation of tumors originating from periosteum, especially of skull, orbits, nasal sinuses, ribs and vertebrae. These locations cause exophthalmus with disturbances of vision. Chloroma is a true cancer having destructive tendencies and forming hematogenous metastases. The sectioned surface of the tumor shows green color due to protoporphyrin. It fades on exposure, can be restored with hydrogen peroxide and preserved by glycerin. There are aleukemic and leukemic forms, the latter with chloroma cells in the blood. Clinically, the latter cases are identical with acute granulocytic leukemia.

Myeloblastoma. This condition differs from chloroma only by absence of pigment.

Eosinophilic Leukemia. Occurs but is extremely rare. Acute and chronic forms have been described with tissue changes similar in every way to those in other forms of leukemia, except that most of the cells in the tissues as well as in the circulating blood were adult differentiated forms of eosinophilic granulocytes. It is difficult or impossible to differentiate this lesion from reactive hyperplastic changes.

Basophilic Leukemia. Still less frequent than eosinophilic but both chronic and acute cases have been described.

Neutrophilic Granulocytic Leukemia. Similar to eosinophilic and basophilic but cell type is the segmented granulocyte, with very few immature forms.

Lymphocytic Sarcoma. Two main forms of this condition are recog-

nized. One begins as a local lesion, and shows later aggressive growth and metastases. This is the form described by Kundrat and known under his name. Cell type is a lymphocyte with nuclear irregularities and variation in size and shape of the whole cell and of the nucleus. It may begin in any part of the body. There are no changes in the blood until late, when anemia may be present, caused by the systemic effect of the malignant disease, by marrow replacement, or, in most cases by hemolytic anemia, frequently associated with autoantibodies. For a discussion of autoantibodies, see Chapter 6. In some cases lymphopenia develops, probably caused by widespread involvement of lymphocytogenic tissues. There are rare cases with a leukemic picture: lymphosarcoma cell leukemia (Sternberg's leukosarcoma). The sarcomatous blood cells show characteristics of cancer cells with nuclear irregularities in shape, size and staining and with several nucleoli. The second form is systemic from the beginning, but otherwise identical with the former. Lymphocytic sarcoma with leukemia is usually a phase of or the end stage in the course of lymphocytic sarcoma.

Erythrocytic sarcoma (synonyms: acute erythremia, erythroleukemia). Clinical picture of acute leukemia. Blood shows erythroblasts, occasional myelocytes and metamyelocytes, but no leukocytosis. At autopsy there is infiltration with immature red blood cells (metarubricytes and less mature forms) in spleen, liver, and an increase of such immature cells in bone marrow.

In addition to erythropoietic, also leukopoietic tissues show hyperplasia.

Mixed Types of Leukemia. Extremely rare cases of combinations of granulocytic and lymphocytic leukemias have been described reliably by good hematologists. However, unless well documented such cases should be accepted very cautiously.

Myeloma. Also known as plasmocytoma. Develops from plasmocellular reticulum cells which are the cell type. Attempts to differentiate lymphocytic, myelocytic, erythroblastic and mixed cell myelomas are not justified. There are occasional leukemic cases with varying numbers of myeloma cells in the circulating blood (see plasmocytic leukemia).

According to Hoff, at the beginning the myeloma is diffuse with generalized plasma cell proliferation in the marrow. He calls this stage aleukemic or leukemic plasma cell leukemia, depending on the blood picture. In due course the neoplastic character becomes manifest in the form of what we call multiple myeloma. The latter shows char-

acteristic metabolic changes. Bone destruction leads to calcium mobilization with increase of calcium in serum and metastatic calcification.

Proteins in the blood are increased, especially globulin. There are variants of the disease differing in the form of globulin which contributes mainly to the rise. They are differentiated as alpha, beta and gamma myelomas or plasmocytomas. The proteins are precipitated in renal tubules. This results in obstruction and elimination of the nephron. Progressive fibrosis, destruction of renal parenchyma and eventually renal insufficiency are the outcome.

LEUKEMOID REACTIONS

Definition

A blood picture resembling leukemia but proven not to be leukemia. Here belongs leukocytosis of 50,000 or higher with a shift to the left or lower counts, even below normal, but with considerable numbers of immature white blood cells and similar quantitative and/or qualitative changes in lymphocytes.

Causes

Congenital: Pelger's congenital cell defect, familial trait, failure of granulocytes to differentiate beyond two segments. The differential count shows occasional myelocytes and metamyelocytes and many band forms.

Neutrophilic Leukemoid Reactions.

1. Hemoclastic crisis in hemolytic anemia. Normoblasts are commonly present in the peripheral blood.
2. Hemorrhage (WBC from 50,000 to 100,000)
3. Hodgkin's disease. Occasional counts over 100,000 with many eosinophils
4. Various infections
   a. Tuberculosis (also lymphocytic leukemoid reactions possible)
   b. Pneumococcus, meningococcus, and streptococcus infections, gas gangrene, diphtheria, leptospirosis, malaria
   c. Syphilis, congenital (also lymphocytic leukemoid reaction possible)
5. Burns
6. Eclampsia
7. Mustard gas poisoning
8. Marrow replacement by tumors including multiple myeloma and myelosclerosis (also eosinophilic, and lymphocytic leukemoid re-

actions possible). Normoblasts are characteristically present in the peripheral blood

Lymphocytic Leukemoid Reaction.

1. Infectious lymphocytosis
2. Infectious mononucleosis
3. Pertussis
4. Varicella

Discussion

Leukemoid blood picture may be seen in a variety of infections for which various infectious agents are responsible. The reaction is irregular, even with the same infectious agent. Hence the conclusion that leukemoid blood pictures are frequently determined by the host reaction. The same micro-organisms in the large majority of cases do not produce that kind of leukemoid blood picture.

Myeloblastic leukemia-like pictures with severe anemia, fever and splenomegaly have especially been seen in tuberculosis with involvement of lymph nodes and spleen.

Differential diagnosis from leukemia is not always easy. Most important is marrow examination, which is more helpful in the differentiation of lymphocytic leukemia than of granulocytic.

Sometimes only prolonged observation may reveal the true nature of the condition, and occasionally the correct diagnosis is not made until the autopsy.

LEUKOPENIA

Definition

A reduction of the total leukocyte count below 5,000.

Causes

Leukopenia is usually due to a drop in the number of granulocytes, because they are numerically the most frequent white blood cells. The life span of granulocytes is only about 2 to 3 days. Anything that interferes with the production of granulocytes will rapidly bring about a leukopenia. Occasionally a lowering of the number of lymphocytes may be responsible.

Leukopenia may be transient as seen frequently in early stages of infectious diseases and of localized severe infections. This has been explained by the depletion of the peripheral circulation of its leukocytes, which concentrate in the area of the infection. As soon as the bone

marrow catches up with the production, the leukopenia is replaced by a leukocytosis.

Discussion

1a. Members of the salmonella group produce a mild initial leukocytosis followed by neutropenia with relative and absolute lymphocytosis, and occasionally also with monocytosis. In typhoid fever there is hyperplasia, especially of the erythropoietic and reticulo-endothelial elements. Focal necrosis may also be present.

b. The leukopenia of measles is usually observed after the appearance of the rash. Blood counts done during the incubation period show a moderate neutrophilic leukocytosis with a shift to the left. The leukopenia persists for about a week. Plasmocytes are frequently seen in the blood smears. The thrombocytes are normal. In rubella, neutropenia and lymphopenia are present from the beginning, and are followed

TABLE 5.18

Causes of Leukopenia

1. Toxic marrow depression:
   (a) bacterial toxins: typhoid, paratyphoid and undulant fever
   (b) viruses: influenza, measles, rubella, mumps, smallpox, dengue fever, psittacosis, etc.
   (c) protozoa: malaria, relapsing fever, kala-azar, etc.
   (d) overwhelming infections, for example, septicemia, miliary tuberculosis
   (e) diseases of liver, for example, infectious hepatitis, portal cirrhosis
   (f) chemicals*
2. Metabolic marrow depression: cachexia. Dietary deficiency
3. Cytotoxic marrow damage: nitrogen mustard, x-ray, radium, benzol, etc.
4. Hypersplenism (splenogenic marrow depression): splenomegaly of any type
5. Autoantibodies
6. Allergic granulocytic shift: chemicals and drugs, barbiturates, etc.
7. Marrow replacement: pernicious anemia, chronic hemolytic anemia, metastatic carcinoma, Gaucher's disease, aplastic anemia
8. Cause undetermined: for example, disseminated lupus erythematosus† and pernicious anemia

* Neutrophilic leukopenia can be produced by amidopyrine, dinitrophenol, organic hair dyes, phenobarbital, quinacrine (atabrine), sulfonamides, trinitrotoluene, tripelennamine hydrochloride (pyribenzamine), neoarsphenamine, potassium arsenate, gold, aprobarbital (alurate), thiouracil, and urethane.

Lymphocytic leukopenia can be produced by nitrogen mustards.

† In terminal stages neutrophilic leukocytosis may supplant leukopenia and is prognostically a bad omen.

before the end of the first week by a lymphocytosis with a relatively high number of plasmocytes (over 10 per cent).

d. Some bacteria, viruses, and protozoa have a direct depressing effect on the bone marrow. In overwhelming infections an effect similar to paralysis seems to be exerted on the bone marrow.

e. The leukopenia seen in diseases of the liver has not been explained satisfactorily. The elimination of the detoxifying function of the liver may play a part.

f. The same drugs which produce a depressing effect may under other circumstances, especially in smaller doses, produce a leukocytosis, and under other circumstances the effect may be directly on the circulating blood and probably of allergic nature.

2. The leukopenia seen in cachexia and in dietary insufficiencies is probably on a metabolic basis. This is suggested by experiments in animals in which the administration of *Lactobacillus casei* factor helps to correct the leukopenia brought about by a protein deficient diet.

3. The direct cytotoxic action of such agents as x-ray, radium and of chemicals such as nitrogen mustard, benzol and others has been demonstrated.

4. The leukopenia of splenomegaly of various types has been attributed to hypersplenism, a hypothetical concept, according to which the spleen exerts a hormonal depressing effect on the bone marrow leading to leukopenia, thrombocytopenia, and anemia, to any one of the three, or to any combination of the three.

5. Evidence has been accumulating that there are antibodies capable of clumping leukocytes of all varieties under proper experimental conditions (leukoagglutinins.) The data thus far gathered permit a few general statments. The substance in the serum responsible for the agglutination of leukocytes has the characteristics of an antibody. It is present in the gamma globulin fraction. Both group specific and nonspecific leukoagglutinins have been described (Moeschlin). In most instances, leukoagglutinins were found in persons with leukopenia, which suggests that they are an autoantibody (Dausset).

6. The disappearance of the granulocytes during allergic reactions is thought to be due to a shift of the cells from the circulating blood to certain areas involved in the allergic reaction.

Leukopenia in allergic reactions is set apart from those due to marrow depression because the leukocytes disappear very rapidly, indicating that it is not due to a marrow depression but to a shift of the circulating granulocytes on an allergic basis.

7. In various conditions such as metastatic carcinoma the leukopenia is due to a replacement of the granulocytogenic areas of the marrow. In Gaucher's disease, in pernicious anemia and in chronic hemolytic anemia, the proliferating cells encroach upon the granulocytic elements. In aplastic anemia the damaging agent, known or unknown, interferes with the normal granulocyte producing ability of the marrow.

8. Finally, there are leukopenias of undetermined origin. Here belongs the leukopenia of lupus erythematosus disseminatus and of pernicious anemia.

The diagnosis and cause of leukopenia can frequently be made by examining the marrow. One may find a hyperplastic, replaced, normal, or hypoplastic marrow. Leukemia, pernicious anemia, various infections (for example kala-azar, malaria) can thus be found.

TEST FOR DISSEMINATED LUPUS ERYTHEMATOSUS (L.E. TEST)

In acute disseminated lupus erythematosus the finding of so-called L.E. (lupus erythematosus) cells can help to make the diagnosis. The L.E. cell is a segmented leukocyte (sometimes two) englulfing one or more large basophilic masses of nuclear material. The inclusions vary in size from less than a red blood cell to several times its size. They are round or oval in shape, purplish blue or brown, granular or homogeneous. The absence of the typical chromatin structure of nuclei is characteristic (see p. 109). Histochemical studies demonstrated depolymerized desoxyribose nucleic acid in the included material. The presence of the L.E. cell supports the diagnosis though it is not absolutely specific for disseminated lupus erythematosus. It has been seen in other conditions. It was demonstrated in patients with penicillin hypersensitivity, and after administration of certain chemotherapeutic agents. The phenomenon is based on the ability of the plasma to stimulate the phagocytic activity of leukocytes. The factor responsible for the L.E. phenomenon is a part of the gamma globulin fraction of the plasma.

From the so-called L.E. cell another cell has to be distinguished. This so-called tart cell can be found in the marrow of apparently normal persons, but more frequently in patients with various lesions seemingly unrelated. The L.E. cell is almost always a mature segmented neutrophilic granulocyte. The nuclear segments surround the amorphous mass. The tart cell contains a second nucleus with a sharp nuclear membrane and distinct structure with sharp separation between chromatin and parachromatin. The second ingested nucleus is usually located in the hollow of the identation of the histiocytic nucleus. Occasionally,

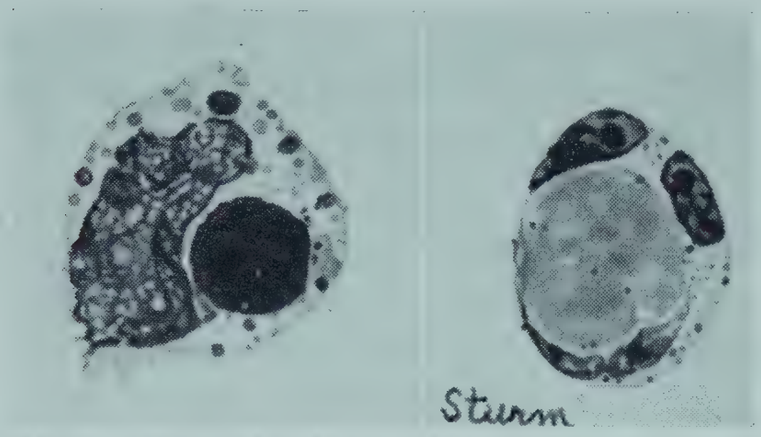


FIG. 5.2. Left: Monocyte with phagocytized pyknotic nucleus (to be differentiated from "L. E." cell). Right: Segmented neutrophil with felt-like purple staining mass in cytoplasm, "L. E." cell. Permission for reproduction from *The Morphology of Blood Cells*, by L. W. Diggs, was obtained from W. B. Saunders Co. and Abbott Laboratories.

two nuclei may be included. The preservation of the nuclear structure helps to differentiate the two cells from each other.

Technic of the L.E. Test

Several technics have been evolved in recent years for the demonstration of the L.E. phenomenon. It is not necessary to employ bone marrow. Buffy coat smears prepared from oxalated blood after incubation at 37°C. for one hour have been recommended. We have had good experience with the technic described by Zimmer and Hargraves:

1. Obtain 10 ml. of venous blood and allow to clot
2. Let blood stand at room temperature for 2 to 4 hours
3. Fragment clot with wooden applicators until serum appears very bloody
4. Remove portions of clot still remaining.
5. Centrifuge the resulting serum-cellular mixture at 2000 r.p.m. for 5 minutes
6. Remove and discard supernatant serum
7. Transfer 1 ml. of top layer of cells to a Wintrobe hematocrit
8. Centrifuge at 2000 r.p.m. for 5 minutes
9. Prepare smears from the concentrated leukocyte layer of cells
10. Stain with Wright stain.

AGRANULOCYTOSIS

Definition. A disease of the myeloid (granulocytogenic) portion of marrow characterized by a marked decrease or complete absence of granulocytes in the peripheral blood.

Clinic. 1. May be acute, subacute, chronic, recurrent. By usage,

agranulocytosis has been commonly considered an acute disease. Because of its nature the term malignant neutropenia has been used.

2. *Symptomatology.* Three phases:
   a. Prodromes with malaise, fever and chills
   b. Onset of agranulocytosis with fatigue, malaise and prostration
   c. Complete agranulocytosis, frequently complicated by secondary bacterial infection of virulent nature.

Severity and progression depend on intensity of toxic agent, rapidity and degree of agranulocytosis, and response of patient (hyperplasia of marrow, etc.). Varies from fulminating to mild chronic and recurrent.

Clinical forms.

a. Acute. Fulminating with severe chills and fever, complete agranulocytosis. No response of patient. Early termination from secondary infection.
b. Subacute. Severe reaction with fever, chills, malaise, prostration. Period of agranulocytosis with secondary infection. Response of patient with marrow hyperplasia and gradual recovery.
c. Chronic or recurrent. Symptoms mild; malaise, dizziness and fatigue. Infection can be controlled with attention and proper antibiotic treatment.
d. Splenic. Splenic enlargement may be associated with pain in region of spleen, fever. Acute or chronic in nature with corresponding prominent or mild symptomatology. Relieved by splenectomy.

Blood.

1. As a rule only agranulocytosis, with normal red cell and platelet count.
2. May be complicated by thrombocytopenia.

Etiology.

1. Various granulocytotoxic drugs.
2. "Splenic" (hypersplenism).
3. Ionizing radiation.
4. Idiopathic.
5. Most important of drugs is amidopyrine.
   a. Causes leukopenia most consistently, with relatively small dosage.
   b. Other drugs not as "toxic" and require greater dosage.
   c. Drug offenders would be more frequently found with adequate history. So-called idiopathic group is probably small.

6. Mode of action.
   a. The benzene ring in the drug is apparently important. However, it is not the only factor and in amidopyrine is not the active factor.
   b. Effect is that of a toxic nature which may be by the mechanism of a hypersensitivity reaction or idiosyncrasy.
   c. Marrow findings: frequently, prominence of immature forms, younger than metamyelocytes; sometimes no apparent changes.

Differential Diagnosis

One of the most important considerations in the differential diagnosis of agranulocytosis is the occasional case which resembles acute granulocytic leukemia. The similarity may be further accentuated by marrow changes similar to those of acute leukemia. There may also be similarity with monocytic leukemia.

The usual conception is that the lesion in agranulocytosis is limited to the granulocytogenic portion of the marrow. There are those who claim that the damage is not limited to the granulocytogenic tissue of the marrow, but because of the short life span of granulocytes (2–3 days) it takes less time for the effect of the damage to become noticeable. It takes weeks before damage to the erythrogenic tissue becomes manifest, because of the long life span of erythrocytes. Thrombocytes have a very short life span, but on the other hand megakaryocytes are highly resistant to damage; and therefore they may be producing thrombocytes for a long time before the damage has advanced sufficiently to interfere with their productivity.

In some cases there is direct damaging or destructive action on granulocytes because they disappear too rapidly from the circulation, which would not be the case if the injury were only at the bone marrow level. When agranulocytosis persists, in many cases there is a drop of thrombocytes. That leads to thrombocytopenic purpura combined with agranulocytosis. Still later anemia may ensue, and pancytopenia results, also known as panmyelophthisis. Then all the formed elements of the blood, red and white cells and thrombocytes, are affected. In some cases the marrow is not damaged, but on the contrary is cell rich or even hyperplastic.

It is possible that the three conditions in which the three marrow elements are involved are not fundamentally different entities but different stages of the same disease.

There is strong evidence in favor of a constitutional factor in agran-

ulocytosis in the form of lessened resistance of the bone marrow to damaging influences (constitutional marrow inferiority). Families have been observed in whom several members have been affected by agranulocytosis and the other conditions mentioned. The existence of dominant hereditary weakness of the granulocytogenic tissues of the marrow has been suggested.

Agranulocytosis, pancytopenia and acute leukemias can all be caused by various agents like x-ray, radium, benzol and similar substances, certain drugs like amidopyrine, arsphenamine, sulfonamides, etc. The reaction to these substances resembles occasionally allergic reactions. The three diseases cannot always be differentiated from each other by marrow changes.

Before the era of marrow punctures the idea prevailed that in agranulocytosis one finds aplasia of granulocytes and of their precursors, in thrombocytopenic purpura damage to the megakaryocytes, and in pancytopenia damage to all myeloid elements in bone marrow. Since then, marrow studies by many authors showed changes which were not at all in accord with the conceptions expressed in the preceding sentence. It is true that sometimes one finds changes similar to those mentioned, but many times findings are entirely different. For example, in agranulocytosis one may find in some cases hypoplasia of granulocytes, in others a hyperplastic marrow with massive precursors of granulocytes, and sometimes, not at all rarely, a myeloblastic marrow identical in every detail with the marrow picture of acute granulocytic leukemia. In some such cases the blood may develop in due course the picture of myeloblastic leukemia and the further course may be one of two: either the patient dies in myeloblastic leukemia, and such cases are recognized as acute granulocytic leukemia preceded by initial agranulocytosis; or the patient gets over the myeloblastic phase, the leukemic gap is closed up, and cure takes place. In the latter case the condition is interpreted as a myeloblastic reaction complicating agranulocytosis.

MYELOID METAPLASIA SYNDROME

A syndrome of circulating immature red and white blood cells, mild anemia and hepatosplenomegaly.

Pathogenesis. a. Replacement of marrow by one of many disease processes, for example, myelosclerosis; damage to marrow by toxic agents; b. compensatory extramedullary hematopoiesis.

Symptoms and Signs. a. Insidious onset with weakness, vague pains, weight loss; b. immature circulating red and white cells out of pro-

portion to anemia; c. marked splenomegaly, moderate hepatomegaly and sometimes adenopathy; myeloid metaplasia in organs.

Marrow. a. Hyperplastic, hypoplastic, or normal; b. functionally inadequate.

HYPERSPLENISM

Primary hypersplenism (primary splenic hematopenia) (Doan). A syndrome of unexplained pancytopenia which may be relieved by the removal of the associated enlarged spleen.

Mechanism. a. Inhibition of cytogenic function of marrow; b. excessive phagocytic activity by spleen.

Clinic. a. Splenomegaly; b. pain in region of spleen.

Laboratory Findings. a. Pancytopenia; b. reticulocytosis and polychromatophilia; c. slightly increased RBC fragility; d. marrow normal or hyperplastic.

Adrenalin Test. Technic of Doan's adrenalin test:

> Patient is kept under basal metabolic conditions for from 15 to 30 minutes (base line). The pulse and blood pressure are recorded and two preliminary complete blood studies are made, and the outlines of the spleen traced. Then from 0.5 to 1 cc. of 1:1,000 adrenalin chloride (depending on the patient's age and vascular integrity) is injected subcutaneously. Blood studies are repeated at 10 minute intervals until the maximum is reached in pulse and blood pressure. This parallels usually the maximum contraction of the spleen. Then peripheral blood studies are continued at 15 minute intervals until the spleen has relaxed.
>
> A rise in the blood cells, especially segmented cells and thrombocytes indicates, according to Doan, that the spleen is responsible.

Inject adrenalin and after noting base line follow contraction of spleen and follow blood counts.

Increased corpuscular elements in blood indicates hypersplenism.

Treatment. Splenectomy.

Secondary Hypersplenism

Similar changes as in primary hypersplenism may be the result of various diseases, all of them producing stasis in, and/or hyperplasia of, splenic pulp, and eventually splenomegaly.

Here belong: portal hypertension following cirrhosis of liver or thrombosis of splenic vein; congestive splenomegaly in chronic cardiac failure; kala-azar; Gaucher's disease; and Hodgkin's disease.

In all these conditions anemia, leukopenia and thrombocytopenia have been seen and have been relieved by splenectomy.

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CHAPTER 6

BLOOD GROUPS. INCLUDING PRETRANSFUSION AND OTHER IMMUNOHEMATOLOGIC TESTS

By I. Davidsohn

THE INDIVIDUALITY OF BLOOD

The human red blood cell is a very complex structure in more ways than one. It shows several morphologic variants in health and a still greater number in disease. Chemical differences have also been recognized. However, the greatest number of differences has been identified in its serologic structure: nine different and independent systems of blood groups. The combinations of these different blood groups and of the still larger number of antigens (blood factors), of which there are several in each blood group system, lead to a very large number of identifiable phenotypes. The number of possible, though not necessarily identifiable genotypes, is greater still. This number is constantly being increased as new blood factors are being discovered. Landsteiner stated once that the time may come when every person's blood will be identifiable as distinct from everybody else's. If one considers that all known blood factors have been discovered during the last 55 years and the majority during the last 15 years, it seems justified to expect that the term "individuality of blood" is approaching reality.

Is individuality a unique attribute of the human red blood cell or do other body cells show a similar degree of extreme differentiation? The experience with tissue and skin transplantations indicates that individuality is not limited to red blood cells, but better methods are available for the demonstration of differences in red cells of different individuals, than for demonstration of tissue differences. There are two main reasons why we have been able to recognize so many serologic variants (groups) of human blood:

1. Hemagglutination technic is a convenient and highly sensitive method which permits the study of the whole cell without destroying or even damaging its identity;
2. Blood transfusion, equivalent to tissue transplantation, is the most frequent of all surgical operations, and it provides a convenient opportunity for the study of serologic and immunologic responses of a delicacy which no other technic would permit.

The nine thus far identified systems of blood groups are:

(1) ABO; (2) MNS; (3) Rh; (4) P; (5) Lewis; (6) Kell; (7) Lutheran; (8) Duffy; (9) Kidd.

They will be presented in as much detail as their clinical significance calls for.

In addition, a few less well studied blood factors have been discovered.

THE ABO SYSTEM

Our knowledge of blood groups is based upon the phenomenon of agglutination of red blood cells by serum or plasma. This special form of agglutination is called hemagglutination. Two factors are necessary for hemagglutination: the red blood cells which are clumped, the passive factor or the agglutinogen, and a substance in the serum or plasma which brings about the clumping, the active factor, the antibody or the agglutinin. Serum which clumps unaltered red cells suspended in saline contains so-called complete antibodies. Recently hemoantibodies have been discovered which combine with the homologous red cells without clumping them. They are called incomplete antibodies.

The injection of red blood cells (the antigen or agglutinogen) of one animal species into another leads to the production of agglutinating antibodies or hemagglutinins. They clump specifically the blood cells of the injected species. Such agglutinating antibodies produced by immunization are called immune agglutinins. Hemagglutinins, not due to known previous immunization, are called natural hemagglutinins. During the last quarter of the 19th century much was written about the agglutination of red cells of one species by the serum of another. The accumulating observations fitted well into the rapidly growing knowledge of differences between species. In 1900 came the fruitful observation by Karl Landsteiner of agglutination of the red cells of one person by the serum of another, which led to the development of our entire knowledge of blood groups. Here for the first time individual differences were observed between blood of healthy and normal members of the same species: iso-agglutination as contrasted with hetero-agglutination or agglutination in mixtures of blood of two different species.

Of the two factors that are essential for hemagglutination one resides in the red blood cell. It is the so-called agglutinogen. There are many of these factors in the red cell. Those that are known have been discovered at various times since 1901. Our knowledge of them is far from complete, and it is likely that more factors will become known in the future.

In his experiments which led to the discovery of blood groups, Landsteiner studied the behavior of mixtures of serum and red cells of different persons. From the behavior of these mixtures he found that people could be divided into three groups: the largest group consisted of persons whose red cells were not clumped by the serum of any other person, the red cells of a large second group and of a small third group were clumped by some serums. He assumed the existence of two different agglutinable substances in the two last mentioned groups, the larger of which became known as group A and the smaller as group B. The absence of the two agglutinable substances from the blood cells which could not be clumped was expressed by the numeral 0. Later it became customary to speak of group (letter) O, instead of group zero.

To these three blood groups Decastello and Sturli added the fourth, the least frequent, by finding a small number of persons whose red cells were clumped by serums of persons belonging to the three other groups: A, B, and O. The red cells of this group contain both factors, A and B, hence the name group AB.

The relation between the agglutinins in the serum and the agglutinogens in the red cells was expressed by Landsteiner in a formula (Landsteiner's law) stating that every person has in his serum the agglutinins which react with the factor or factors absent from his red cells.

The iso-agglutinins in serum of group A react with red cells of group B and are called anti-B. The iso-agglutinins in serum B are called anti-A because they react with red cells A. In serum of group O both agglutinins, anti-A and anti-B, are present because the red cells of this group have no agglutinogens. Conversely no agglutinins are present in serum of group AB because both A and B factors are present in the red cells. One finds in the literature also designations a and b, and sometimes Greek letters α and β for the iso-agglutinins, but anti-A and anti-B are more descriptive and less subject to misunderstanding, and will be used in this chapter.

In the process of clumping the A and B factors unite with the corresponding iso-agglutinins and if the test is done in a test tube and the clumped cells permitted to settle, the supernatant fluid will be found to have lost its agglutinating properties: the agglutinins were absorbed. There are variations in the ability of red cells of different persons to absorb agglutinins. The quantity of the red cells and the titer of the iso-agglutinins are the other factors which determine the absorption or neutralization of iso-agglutinins.

From the preceding it is apparent that a blood group is characterized

by properties of red cells and of serum. This is expressed as blood group formulas: A anti-B, B anti-A, O anti-A, anti-B, and AB.

Much has been learned about the four blood groups during the last fifty-five years. They are detectable in early fetal life, persist all through life, and resist destruction in the dead body for a long time. They are not related to sex or any body characteristics. They are not influenced by disease, but they may be responsible for blood transfusion reactions and for hemolytic disease of the newborn, as will be presented later.

Subgroups of Group A

Factor A, present in groups A and AB, was shown quite early by Dungern and Hirschfeld to consist of two fractions, now known as A_1 and A_2, thus leading to the recognition of four subgroups A_1, A_2, A_1B and A_2B. When a serum with anti-A agglutinins, for instance, a serum of group B, is mixed with red cells of ten or more persons of group A, there will be one or two among them whose cells will clump conspicuously less vigorously and slower than those of the majority. These are the cells of subgroup A_2. They constitute roughly one-fifth of all persons of group A. The strongly agglutinating cells are those of subgroup A_1 which is present in about 80 per cent of persons of group A. Group AB is divided into subgroup A_1B (about 60 per cent of group AB) with a strongly agglutinable A, and subgroup A_2B (about 40 per cent of group AB) with a weakly agglutinable A. The weaker agglutinability of A_2 parallels its lesser ability to absorb anti-A agglutinins: whereas, as little as 1/32 volume of packed red cells of subgroup A_1 will remove the anti-A iso-agglutinins from a volume of serum B; even 1/2 volume of cells A_2 will, as a rule, fail to accomplish it.

The difference between the subgroups is not merely quantitative, a little more or a little less of the same factor A. There is a qualitative difference, as shown by the fact that in some persons of one subgroup there may be found agglutinins for the red cells of the other subgroup. The rare agglutinin in subgroups A_2 and A_2B is known as anti-A_1. It clumps specifically red cells of subgroups A_1 and A_1B. The still less frequent agglutinin in subgroups A_1 and A_1B is known as anti-A_2. It clumps specifically red cells of subgroups A_2 and A_2B. Anti-A_1 occurs, usually in small amounts, in serums of from 25 to 30 per cent of subgroup A_2B and in about 2 per cent of serums of subgroups A_2. Only about 1 out of 250 of subgroup A_1 has the irregular agglutinin anti-A_2. Its formula is then A_1 anti-B, anti-A_2. These irregular agglutinins react best at low temperatures (up to about 15°–18° C.), but sometimes their ability to clump extends to body temperature. They are of interest mainly because they may be the source of confusion and errors in blood grouping and crossmatching. It is a noteworthy feature of the anti-A_2

agglutinin that it reacts not only with red cells of subgroups A_2 and A_2B but also with cells of group O which are known to be otherwise not agglutinable by normal human serum. The practical consequence of this is another possibility of confusion, especially in crossmatching tests. Another consequence of the discovery of the anti-A_2 agglutinin was that it made it necessary to abandon the former idea that red cells of group O are devoid of iso-agglutinogens.

A consideration of the subgroups leads to the following formulas: A_1 anti-B, with infrequent presence of anti-A_2 (anti-O); A_2 (anti-B), with an occasional anti-A_1; A_1B, with an infrequent anti-A_2 (anti-O) and A_2B, with a not infrequent anti-A_1. See Table 6.1 for presentation of the relations discussed in the preceding paragraphs.

A third variety of the property A still weaker than A_2 was described by Friedenreich as A_3. It is rare but seems to be a definite entity, and its main significance rests in its extremely feeble agglutinability. Unless a strong typing serum is used, red cells of this subgroup may be passed up as lacking agglutinability and be accepted as being of group O.

Classification of the ABO Blood Factors

Until recently, three different classifications have been in common use. In an attempt to correct the resulting confusion, Landsteiner's classification in which the groups are named according to the agglutinogen content has been recommended for adoption. This classification as well as those of Jansky and of Moss are given in Table 6.1.

Jansky and Moss, mentioned in the preceding paragraph, are the authors of two classifications of blood groups employing numbers and known under their names. They did not know of each others' recommendations and used opposite numbers for groups AB and O, which led to confusion. For that reason the Landsteiner or International classification is preferable.

The AB Factors in Tissues and Secretions

The terms A and B blood factors are misnomers since it was found that they are not limited to red blood cells but are present in tissue cells of all persons and in secretions of most. Those who have the group substances in their secretions (about 80 per cent) are called secretors, the others, a minority, non-secretors. Some tissues of secretors, for instance, the pancreas contain water-soluble group substances in very large amounts. The same holds for secretions like saliva and gastric juice. It was shown that a substance of ferment-like nature rapidly destroys the A and B specific substances in secretions. Placing saliva in a boiling water bath for from 10 to 20 minutes destroys the enzyme but does not affect the thermostable A and B substances.

TABLE 6.1

The ABO System

Factors in Red Blood Cells								Iso-agglutinins in Serum	
Classification				Genotype	Reactions with typing serums			Normal	Irregular
Jansky	Moss	International Phenotype	Subgroups		Anti-A	Anti-A_1	Anti-B		
IV	I	AB (so-called universal recipient) (4%)	A_1B (60%)	A_1B	+	+	+	None	Anti-A_2 = Anti-O very rare. Reacts with 95% A_2 and with all O cells
			A_2B (40%)	A_2B	+	−	+	None	Anti-A_1. Present in about 30%. Reacts with A_1 and A_1B cells
II	II	A (41%)	A_1 (80%)	A_1A_1	+	+	−	Anti-B	Anti-A_2 = Anti-O very rare. Reacts with 95% A_2 and with all O cells
				A_1A_2	+	+	−		
				A_1O	+	+	−		
			A_2 (20%)	A_2A_2	+	−	−	Anti-B	Anti-A_1 present in 1 to 2% of cases. Reacts with A_1 and A_1B cells
				A_2O	+	−	−		
III	III	B (10%)		BB	−	−	+	Anti-A, Anti-A_1	Anti-A_2 = Anti-O very rare. Reacts with 95% of A cells and with all O cells
				BO	−	−	+		
I	IV	O (so-called universal donor) (45%)		OO	−	−	−	Anti-A, Anti-A_1, Anti-B	

The difference between secretors and non-secretors is due to production of two different substances. The group-specific substances occur in the human body in two forms: (1) soluble in alcohol, chloroform, and similar lipoid solvents, (2) water soluble. The alcohol-soluble form is present in erythrocytes and almost all tissues (except brain) of every person. It is not present in secretions. The water-soluble form is absent in red cells, but present in the tissues and most body fluids, especially in glandular organs of the majority of persons. The latter are the secretors, the former the non-secretors. The glandular organs manufacture the water-soluble group specific substance. From 74 to 86 per cent of the population are secretors and from 14 to 26 per cent are nonsecretors. The ability to secrete is inherited as a Mendelian dominant.

The ability to secrete is best tested in the saliva. The presence of the A and B factors in the saliva is determined by testing the titer of anti-A and anti-B agglutinins in a serum, then adding saliva to the serum and retesting the titer of the agglutinins after the saliva was permitted to act on the serum. If the titer remains essentially unchanged, there is no antigen present in the saliva, and vice versa.

Blood group specific substances, similar or identical with human A and B, are present in many animal species. This has found a practical application by permitting preparation of purified group specific substances which have been used successfully for neutralization of isoagglutinins, especially in the blood of so-called universal donors. The A substance is extracted from the gastric mucosa of hogs and the B substance from the gastric mucosa of horses.

ISO-AGGLUTININS

Iso-agglutinins can be divided into several classes:

I. Natural regular iso-agglutinins[1]

(1) Anti-A in serums of groups B and O.

(2) Anti-B in serums of groups A and O.

Both have the widest range of thermal reactivity of all iso-agglutinins, being able to clump the corresponding red cells at icebox, room, and body temperatures and up to about 50°C.; they react strongest at the lower temperatures and lose their strength gradually as the temperature rises.

[1] The term regular is used to mean: occurring regularly and fitting into Landsteiner's scheme of blood group agglutinogens and agglutinins.

II. Natural irregular iso-agglutinins[2]

(1) Anti-A_1 found in serums of subgroups A_2 (in from 1 to 2 per cent) and A_2B (in about 26 per cent).

(2) Anti-A_2 and anti-O found very rarely in serums of subgroups A_1 and A_1B.

Both react best at lower temperatures up to about 15°–18°C., but occasionally they may react even at body temperature. Both though listed as natural, i.e., non-immune iso-agglutinins, appear sometimes following blood transfusions. It is possible that their titer then rises from subthreshold to detectable levels.

Irregular iso-agglutinins of all kinds have as a rule a low titer and are less thermoresistant than regular iso-agglutinins.

(3) Anti-M. Extremely rare.

(4) Anti-N. Only a few cases reported.

III. Immune Rh antibodies and other immune antibodies (see p. 330).

IV. Cold agglutinins.

They are non-specific agglutinins, i.e., they clump red cells regardless of blood group, even those of the same person. They react strongest and frequently only at icebox temperature, occasionally at somewhat higher temperatures (20°C. and higher), and then show sometimes a tendency to selective reactivity by agglutinating red cells of some persons and not of others. This widening of thermal range and selective reactivity is seen more frequently in patients with infections and may be the product of immunization.

THE MNS SYSTEM

The next blood factors, chronologically, are M and N. They were discovered by Landsteiner and Levine in 1927. There are no agglutinins reacting with them in normal human blood. That is the reason why they and the other factors to be discussed have been recognized later than A, B, and O. Landsteiner and Levine discovered them in the following way. They injected rabbits with human blood O. As was to be expected the serum of the rabbits clumped red cells of all groups, but when the agglutinins in a sample of serum were absorbed with cells of one person, and then centrifuged, the supernatant serum failed to clump the cells of some persons but did clump the cells of other persons, regardless of the blood group. This suggested the existence of differences between red cells of group O. A number of such immunization

[2] The term irregular is used here to mean that they are not found regularly in the A B O group system.

and absorption experiments established the existence in human red cells of two new factors which were named M and N. They are permanent properties of blood, present in the young fetus, fully developed at birth and persisting through life, not influenced by diseases or environment, and not related to sex. They are independent of factors A, B, and O, which means that their frequency is the same in all four blood groups. One of them or both together are present in the blood of every person. This is different than in the case of the factors A and B, both of which are absent from the blood of more than 40 per cent of the population in some parts of the world. Based on their presence there are three classes of blood called blood types M, N, and MN occurring in about 30, 20 and 50 per cent of the Caucasoid population respectively. There is another difference between blood group factors A and B and blood type factors M and N, in addition to the absence of normal agglutinins in human serum: M and N are found in the red cells and various tissues, but not in body fluids and secretions, while A and B are present also in secretions and in almost all body fluids, except the cerebrospinal fluid.

M and N have been found in younger fetuses than A and B, and they are fully developed in the newborn infant, while A and B factors are considerably weaker in the newborn than later and gradually gain in strength. This quantitative change is measured by the ability of the red cells to absorb corresponding iso-agglutinins.

A new blood factor S was discovered in 1947. It is linked genetically to M and N. This new factor was discovered with the help of a serum which divided the M, N and MN types into two classes: S-positive (S) (55 per cent) and S-negative (s) (45 per cent). With the help of three serums: anti-M, anti-N, and anti-S, one can distinguish the following six types in the MNS system: MS (with two genotypes MS/MS and MS/Ms); Ms (genotype Ms/Ms); MNS (genotypes MS/NS, MS/Ns, Ms/NS); MNs (genotype Ms/Ns); NS (genotypes NS/NS, NS/Ns); Ns (genotype Ns/Ns). There is no anti-s serum available, therefore one cannot at the present time distinguish the SS genotype from the Ss, except by family studies.

There are two reasons why M, N, and S factors need not be determined prior to blood transfusions: normal agglutinins reacting with M or N are extremely rare, and it seems that they are formed just as seldom when blood of type M or N is injected into a person who does not have it. Only few cases of anti-M agglutinins have thus far been reported, three following blood transfusion (Davidsohn and Schirmer, Wiener and Forer). Some of these reacted only feebly and more distinctly at lower temperatures. There is on record a case of a trans-

fusion reaction due to anti-M agglutinins (Broman). Anti-N agglutinins have been observed by Singer in a person of type M following several transfusions of blood N. Several examples of anti-S agglutinins have been seen in S-negative persons immunized with S-positive blood. They do not occur naturally and have not been produced in animals. They have been found responsible for hemolytic transfusion reactions.

The application of the M and N factors in medical jurisprudence will be discussed later.

THE Rh SYSTEM

The so-called Rh factor was discovered in 1940 by Landsteiner and Wiener. They injected rabbits with blood cells of the rhesus monkey (Macaca rhesus). Following a series of injections the serum of the rabbits clumped not only the cells of the monkey but the cells of about 85 per cent of human beings independently of their other blood groups. Thus was discovered a new blood factor, common to some persons and to the rhesus monkey, the rhesus factor or as the discoverers chose to call it, the Rh factor, using the first two letters of the name of the monkey. Some of its properties are similar to those possessed by factors A and B, and some to those of M and N. It is inherited as a Mendelian dominant and has the ability to produce hetero-agglutinins when injected into animals just as all the other blood factors. It was thought at first that the Rh factor is found only in the red cells, but recently its presence was detected also in tissues and in the amniotic fluid, but not in other secretions, thus resembling M and N but not A and B. It was found in a fetus of 17 cm. and is readily demonstrable at birth. The absence of normal iso-agglutinins in serum likens the Rh factor to M and N, but unlike the latter, injection of blood with the Rh factor (Rh-positive blood) into a person without it (Rh-negative) stimulates the production of anti-Rh agglutinins. This is the reason which makes the Rh factor important in connection with blood transfusions.

The discovery of several types of the Rh factor in a relatively short time and the use of a fairly complicated terminology has led to much confusion which can be best cleared up by a little attention to a few basic facts regarding nomenclature.

Table 6.2 gives, side by side, the two most prevalent systems of nomenclature. According to some writers the Fisher-Race nomenclature is easier to teach. On the other hand there is no convincing scientific argument in favor of either of the two systems, and Wiener's comprehensive abbreviations are convenient, at least in conversation.

The Fisher-Race terminology seems more precise, but some of the precision is based on differences which cannot be demonstrated in every case because at least one testing serum is at present not yet available.

The Wiener Nomenclature

Eight different Rh types are recognized, according to presence of one or more of the Rh factors. All Rh types containing the Rh_0 factor are designated with "Capital Rh", or with "Capital R" when the gene is referred to. Combinations of Rh_0 with rh′ or rh″ lead to the designation of Rh_1 or Rh_2, respectively. The corresponding genes for Rh_0, Rh_1, and Rh_2 are represented by R^0, R^1, R^2. Rh_1Rh_2 signifies presence of three Rh factors: Rh_0, rh′, and rh″. The corresponding gene is expressed as R^1R^2, or R^z. In the absence of the Rh_0 factor, the following types may be found (corresponding genes are put in parentheses): rh (r), rh′ (r′), rh″ (r″), rh′rh″ (r′r″ or r^y).

The Fisher-Race Nomenclature

Of the six antigens, CDE and cde, those expressed in small letters are allelomorphs of the corresponding antigens designated with the large letters. There are three sets of allelic genes: C and c; D and d; E and e; and three closely linked loci on a pair of chromosomes, one locus for each set of genes. C or c, D or d, and E or e, are carried on the same chromosome. There is no dominance among these genes; all behave as though they were Mendelian dominants. Of the pair of chromosomes which carry the Rh genes, each carries three genes which can be assembled in eight different ways: cde, Cde, cdE, CdE, cDe, CDe, cDE, CDE.

Gene D is antigenically the most potent. It is present in about 85 per cent of the white population.

Explanatory Notes to Table 6.2

Table 6.2 presents in the first column the *8 types of the Rh factor*, to one of which everyone must belong. Both Wiener's and the British (Fisher-Race) nomenclature are given.

The second column indicates the *incidence* of these types, as established by typing of the North American Caucasoid population.

The next three columns refer to the *3 Rh typing serums* and indicate the results obtained with these serums: + stands for clumping, − for absence of clumping.

The 8 Rh types listed in column 1 are called *phenotypes*, which in the terms of genetics indicates the apparent or dominant qualities (such as color of eyes, hair, etc.) without indicating the masked or recessive qualities, which may reappear in the offspring according to the Mendelian laws of inheritance.

TABLE 6.2

Serologic Differentiation of Rh Types

Phenotypes			Anti-Rh (anti-CDE) Testing Serums*			Genotypes		Anti-Hr (anti-cde) Testing Serums*			
Nomenclature		Approx. freq.‡	70% W/B anti-rh′/C	85% W/B anti-Rh₀/D	30% W/B anti-rh″/E	W	B	80% W/B anti-hr′/c	65% W/B anti-Hr₀/d§	96% W/B anti-hr″/e	Approx. freq.‡
W†	B†										
rh	cde	13.4	−	−	−	1. rr	cde/cde	(+)	(+)	(+)	13.4
rh′	Cde	1.1	+	−	−	2. r′r′	Cde/Cde	−	(+)	(+)	0.02
						3. r′r	Cde/cde	+	(+)	(+)	1.1
rh″	cdE	0.4	−	−	+	4. r″r″	cdE/cdE	(+)	(+)	−	0.003
						5. r″r	cdE/cde	(+)	(+)	+	0.4
rh′rh″	CdE	0.02	+	−	+	6. r^yr^y	CdE/CdE	−	(+)	−	0.000001
						7. r^yr'	CdE/Cde	−	(+)	+	0.0003
						8. r^yr''	CdE/cdE	+	(+)	−	0.0001
						9. r′r″	Cde/cdE¶	+	(+)	+	0.02
						10. r^yr	CdE/cde¶				
Rh_0	cDe	2.5	−	+	−	11. R^0R^0	cDe/cDe	(+)	−	(+)	0.1
						12. R^0r	cDe/cde	(+)	+	(+)	2.4
Rh_1	CDe	51.2	+	+	−	13. R^1R^1	CDe/CDe	−	−	(+)	16.9
						14. R^1r'	CDe/Cde	−	+	(+)	1.1
						15. R^1R^0	CDe/cDe	+	−	(+)	2.6
						16. R^1r	CDe/cde	+	+	(+)	30.6
						17. R^0r'	cDe/Cde				

Rh_2	cDE	16.5	−	+	+	18. R^2R^2	cDE/cDE	(+)	−	−	2.7
						19. R^2r''	cDE/cdE	(+)	+	−	0.2
						20. R^2R^0	cDE/cDe	(+)	−	+	1.1
						21. R^2r 22. R^0r''	cDE/cde cDe/cdE	(+)	+	+	12.5
Rh_1Rh_2	CDE	14.9	+	+	+	23. R^zR^z	CDE/CDE	−	−	−	0.0001
						24. R^zR^1	CDE/CDe	−	−	+	0.08
						25. R^zR^2	CDE/cDE	+	−	−	0.03
						26. R^zr^y	CDE/CdE	−	+	−	0.00002
						27. R^zR^0 28. R^1R^2	CDE/cDe CDe/cDE	+	−	+	13.9
						29. R^zr' 30. R^1r^y	CDE/Cde CDe/CdE	−	+	+	0.001
						31. R^zr'' 32. R^2r^y	CDE/cdE cDE/CdE	+	+	−	0.0004
						33. R^zr 34. R^0r^y 35. R^1r'' 36. R^2r'	CDE/cde cDe/CdE CDe/cdE cDE/Cde	+	+	+	0.9

* Percentage figures for the testing serums indicate the numbers that would be clumped with each serum in 100 specimens collected at random.

† W, Wiener's nomenclature, B, British nomenclature.

‡ After Wiener, A. S.: Rh-Hr Blood Types. New York, Grune & Stratton, 1954, p. 358.

§ Anti-Hr_0 (anti-d) testing serum is as yet not available.

¶ Genotypes which are bracketed cannot be distinguished from each other by means of serologic tests.

+, agglutination, −, no agglutination, (+), agglutination invariably present, and hence test need not be done.

In the seventh column the 8 Rh types are subdivided into the *genotypes* which may occur in each of them. The genotype indicates the genetic make-up of an individual in regard to a certain quality: each inherited quality is due to the presence of two genetic units, called allelomorphs. If both allelomorphs are identical, the individual is *homozygous* for this quality. If the allelomorphs are different, he is *heterozygous*.

Homozygous individuals always transmit the respective quality to their offspring, while heterozygous individuals do so only in approximately half of the offspring. Hence the significance in clinical medicine of determining the homozygosity or heterozygosity of an individual (mainly in cases of Rh-positive husbands of pregnant Rh-negative women).

One method of establishing *proof for heterozygosity* is to determine the Rh type in both the parents and in the offspring of an individual: if any one of them is found to be Rh-negative, the person is heterozygous Rh-positive. Therefore he has in his make-up also the Rh-negative allelomorph, and may transmit this to about half of his offspring.

Homo- and heterozygosity of Rh_1 individuals for the rh′(C) factor may also be determined with anti-hr′ (anti-c) serums: red cells of heterozygous Rh_1 persons are agglutinated, cells of homozygous Rh_1 persons are not.

The eighth, ninth and tenth columns refer to the typing serums for the three Hr (cde) types. Agglutination occurs whenever cells containing the *small* letters (c, d, e) in the British nomenclature meet with the corresponding antiserum.

(+) indicates agglutination which must occur invariably on the basis of the tests listed under Rh typing serums; hence such tests need not be repeated with Hr typing serums. As stated in footnote §, anti-Hr_0 (anti-d) serum is not available.

A number of genotypes—connected with each other by brackets—give identical results with anti-Hr serums, because the serum cannot differentiate whether the antigen appears on the left or right side of the genotype. This represents the limitation of serologic differentiation of Rh genotypes. In these cases only genetic studies may decide for one of the possible alternatives.

The last column lists the approximate frequencies of the 36 Rh genotypes in the U. S. Caucasoid population.

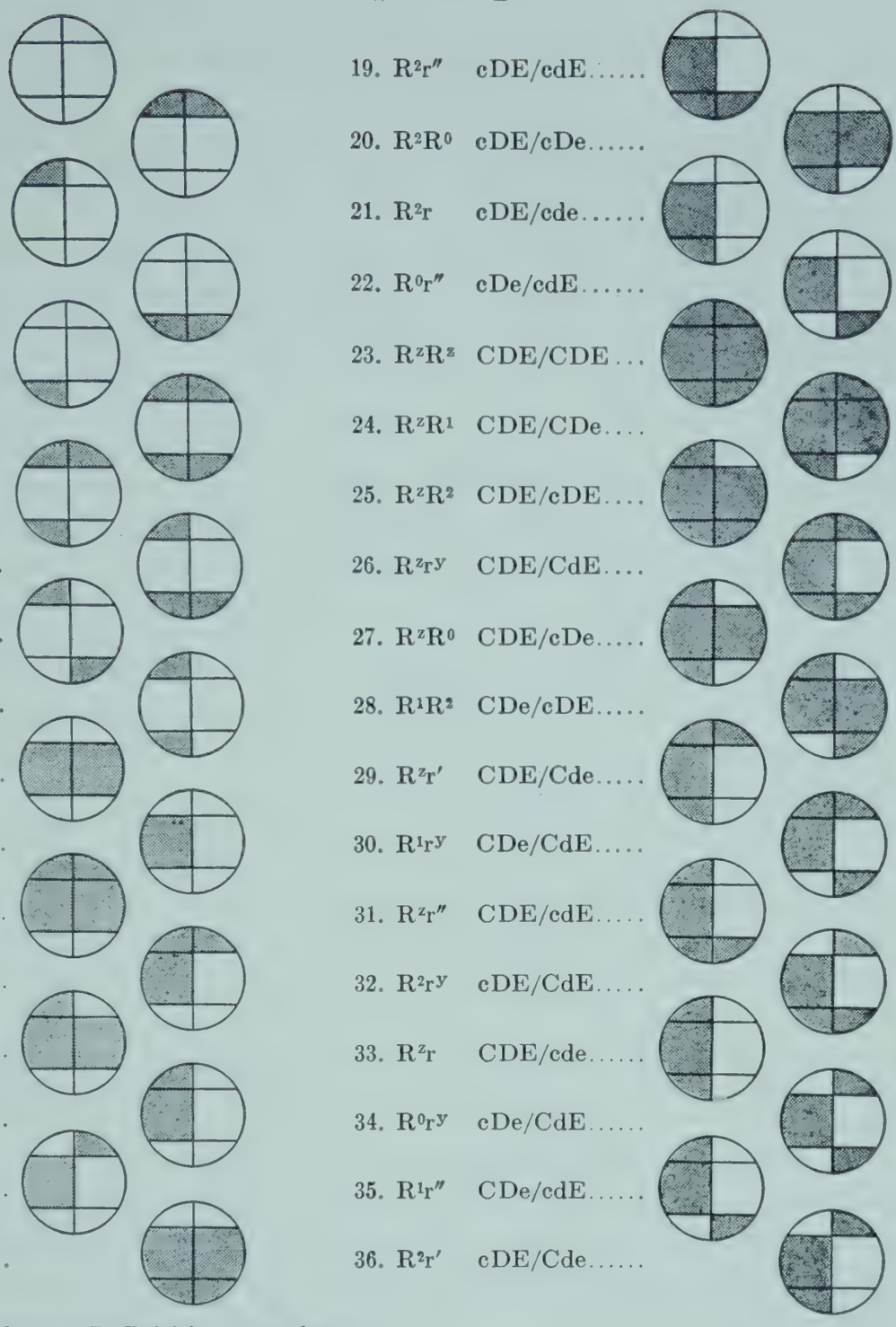
These antigens are detected with immune serums found in mothers of erythroblastotic babies, in individuals who have received repeated transfusions of incompatible blood, or in volunteers immunized for that purpose.

The typing serums, their names in both systems of terminology and the frequency of their reactions are stated in Table 6.2. Of these serums anti-d is not yet available. The frequency with which these serums react with samples of blood collected at random corresponds with the distribution of the corresponding antigen in the population. Due to racial differences in the incidence of these antigens, the frequency of reactions with these serums will vary in different parts of the world and in different ethnic groups.

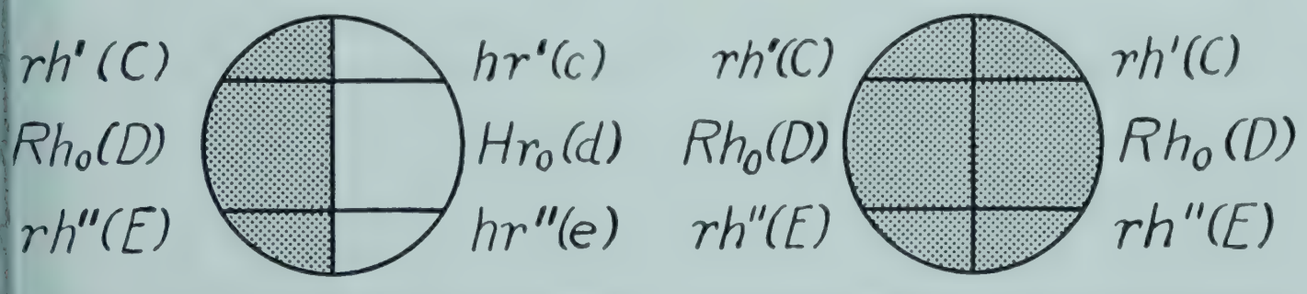
As seen in Table 6.2 several combinations of genes are possible in each phenotype. The possible genotypes are listed in Fig. 6.1. For practical reasons the

W*	B*
. rr	cde/cde.....
. r′r′	Cde/Cde.....
. r′r	Cde/cde.....
. r″r″	cdE/cdE.....
. r″r	cdE/cde.....
. r^yr^y	CdE/CdE.....
. r^yr'	CdE/Cde.....
. r^yr''	CdE/cdE.....
. r′r″	Cde/cdE.....
. r^yr	CdE/cde.....
. R^0R^0	cDe/cDe.....
. R^0r	cDe/cde.....
. R^1R^1	CDe/CDe.....
. R^1r'	CDe/Cde.....
. R^1R^0	CDe/cDe.....
. R^1r	CDe/cde.....
. R^0r'	cDe/Cde.....
. R^2R^2	cDE/cDE.....

W*	B*
19. R^2r''	cDE/cdE.....
20. R^2R^0	cDE/cDe.....
21. R^2r	cDE/cde.....
22. R^0r''	cDe/cdE.....
23. R^zR^z	CDE/CDE...
24. R^zR^1	CDE/CDe....
25. R^zR^2	CDE/cDE....
26. R^zr^y	CDE/CdE....
27. R^zR^0	CDE/cDe.....
28. R^1R^2	CDe/cDE.....
29. R^zr'	CDE/Cde.....
30. R^1r^y	CDe/CdE.....
31. R^zr''	CDE/cdE.....
32. R^2r^y	cDE/CdE.....
33. R^zr	CDE/cde.....
34. R^0r^y	cDe/CdE.....
35. R^1r''	CDe/cdE.....
36. R^2r'	cDE/Cde.....



* W, Wiener's nomenclature; B, British nomenclature.



Each circle indicates the combination of Rh-Hr antigens in one of the 36 Rh genotypes.

Left and right halves of the circle represent the two alleles. Symmetry indicates homozygosity, assymetry heterozygosity.

Presence of Rh antigens is indicated as shaded; presence of Hr antigens as white space.

The upper segment represents either rh′ or hr′; the middle segment, Rh_0 or Hr_0; the lower segment, rh″ or hr″.

FIG. 6.1. Symbols of Rh genotypes
(This figure was designed by Dr. Kurt Stern)

most common genotype in a group is used as the name of the type. For example, red cells of the most common type, Rh_1, are separated into two main divisions, depending on the homozygosity of gene C. Those that give a positive reaction with anti-c are considered heterozygous Rh_1. The most frequent representative in this heterozygous group is genotype R^1r (in Wiener's nomenclature), or CDe/cde (in Fisher-Race's nomenclature), with a calculated frequency of 30.6. In the same group and indistinguishable from the former by means of anti-c, is type CDe/cDe (R^1R^0) (frequency 2.6). The distinction could be made with anti-d, which is not available. Even after anti-d will become available it will not be possible to distinguish all types, for example, CDe/cde (R^1r) from Cde/cDe ($r'R^0$). In practice the error is small because the calculated frequency of the latter type is only 0.05 per cent. This problem may be solved in some cases with the help of new agglutinating serums capable of detecting further antigenic differences, some known already, and some no doubt awaiting discovery.

The practical clinical application of genotyping is mainly for the purpose of prognosis in evaluating the outlook of recurrence of fetal erythroblastosis. It is important to realize its limitations. Since D is the most potent antigen, the main purpose of genotyping is to detect the presence of D in the husband's blood. The finding that a husband's blood of type Rh_1 is homozygous for C renders a falsely bad prognosis in 1 out of 22 cases because the D homozygous CDe/CDe (R^1R^1) cannot be distinguished, as a rule, from the D heterozygous CDe/Cde (R^1r') without anti-d. Similarly a falsely hopeful prognosis once in 16 cases will be rendered when a husband of the same type is found to be heterozygous, because CDe/cDe (R^1R^0) cannot, as a rule, be distinguished from CDe/cde (R^1r) except with anti-d. Fortunately even now without the anti-d, some such problems can be solved by typing the parents of the father and all his available children.

In addition to the differences in nomenclature the other essential argument between Wiener on one hand and Race and Fisher on the other is whether or not there are three closely linked genes on three loci (Race and Fisher) or multiple allelomorphs at a single locus (Wiener). This argument can be best left to geneticists. The eventual solution one way or another will have little or no clinical significance.

In addition to the six genes previously listed further alleles named C^w and D^u have been discovered at the Cc and Dd loci. D^u is considered to be a mutation from D and not from d.

The term D^u is a collective designation which includes a number of variants of D comprising about 1 per cent of all D-positive persons. They are recognized by being clumped by some anti-D serums and not by others. The close relation of D^u to D is shown by the fact that immunization with D sometimes produces an immune serum which reacts with D and with D^u. The relation between C and C^w is manifested by the fact that immunization with C produces in about 50 per cent of cases antibodies against C and C^w. However, serologically and genetically D^u is a distinct variant of D, and C^w of C.

C, C^w and c are Mendelian allelomorphs, giving six genotypes: CC, CC^w, Cc, C^wC, C^wc, cc.

Recently (1953) a new Rh type was described, labeled as f. The antibody anti-f was found in the serum of a man who received many transfusions. About 66 per cent of white people have this new blood factor.

Rh_0 (D) is the only really potent antigen in the Rh system capable of mmunizing from 50 to 75 per cent of Rh_0-negative (D-negative) recip-ents of Rh_0-positive (D-positive) blood given by transfusion, but only per cent of Rh_0-negative (D-negative) women are sensitized by preg-ancies. Sensitization by the other antigens of the Rh system is rare.

Rh Antibodies

In the ABO system antibodies or iso-agglutinins against the absent ntigen are present naturally and rise further on immunization. In the MNS system there are, as a rule, no natural antibodies and none are roduced artificially (except on rarest occasions). In the Rh system here are no natural antibodies, but they can be produced by parenteral mmunization (transfusions or pregnancy). There are as many varie-ies of Rh antibodies as there are Rh antigens: anti-Rh_0 (anti-D), anti-h′ (anti-C), anti-rh″ (anti-E), anti-hr′ (anti-c), anti-hr″ (anti-e), anti-C^w and anti-D^u. Anti-Hr_0 (anti-d) is not yet available. In table 6.2 he reactivity of these type specific serums is stated. They are obtain-ble from three sources: (1) persons sensitized by transfusions with in-ompatible blood; (2) women who become sensitized in the course of regnancies; (3) persons (volunteers) immunized intentionally.

In addition to differences in Rh antibodies based on type specificity, lifferences have been found which are manifested by altered reactivity lepending on the set-up of the test and on the physico-chemical make-up f the diluent. The fact that different technics are used for detection f the antibody does not mean that the antibodies are qualitatively lifferent. It may be that varying degrees of sensitization and/or pro-onged immunization are involved.

Some type specific antiserums react when physiologic saline is used as liluent, hence the name saline agglutinins (see Table 6.3). Only from 0 to 80 per cent of mothers of infants with erythroblastosis showed erologic evidence of sensitization in the form of antibodies when the ld method of testing was used with saline as diluent for the serum and s menstruum for the red cell suspension. This technic detects the o-called saline agglutinins (bivalent agglutinins of Wiener). This anti-ody is active in saline, but unlike most other blood group antibodies, t is more active at 37°C. than at lower temperatures. It can be ab-orbed by red cells and eluted from red cells.

Incomplete Antibodies

There are four other technics for detection of Rh antibodies. In one f them the antibody combines with the red cell antigen but does not

TABLE 6.3

Types of Rh Antibodies

	Nomenclature	Action	Occurrence	Possible Clinical Significance
	A. Complete Antibodies			
I	Saline agglutinins Bivalent agglutinins (Wiener) Thermolabile agglutinins (Diamond)	Clump Rh-positive cells in saline medium	As a rule, present together with serum-albumin agglutinins, but are never found together with blocking antibodies in the same serum	Appear in the early stages of sensitization. Presence in pregnant women: chances of survival of infant relatively good
	B. Incomplete Antibodies (Race)			
II*	Serum-albumin agglutinins Glutinins (Wiener) Univalent agglutinins (Wiener) Thermostabile agglutinins (Diamond)	Clump Rh-positive red cells in medium containing albumin or similar macro-molecular colloids. React in the indirect Coombs test and with enzyme-treated red cells.	May be found: (a) alone; (b) together with saline agglutinins; (c) together with blocking antibodies	May be expression of higher degree of sensitization. Elevated titers during pregnancy affect prognosis of infant adversely
III*	Blocking antibodies (Wiener)	Unite with Rh-positive cells without manifest clumping, and prevent cells to which they become attached from being clumped by Rh antibodies of saline agglutinin type	As a rule, occur together with serum-albumin agglutinins; are never found together with saline agglutinins	Are expression of high degree of sensitization (hyper-immune antibodies, Diamond). If present during pregnancy, are followed frequently by stillbirth or hydrops. Are found frequently in pregnant women, who received Rh-positive blood transfusion prior to pregnancy

roduce visible clumping. The union with red cells can be demon-trated by the fact that addition of a serum with potent saline agglu-inins does not cause clumping: the surface of the red cells is coated vith incomplete antibody, which blocks the action of saline agglu-inins, hence the more popular name "blocking antibody." Blocking ntibody is specific for the Rh_0 (D) antigen and of low sensitivity.

Incomplete antibody is more thermo-resistant than saline agglutinin, also more esistant to chemicals, for example, merthiolate. Photo-oxidation inactivated nti-Rh agglutinins but did not change them into incomplete anti-Rh. High pres-ure had the same effect. Incomplete Rh antibody resisted pressures which nactivated agglutinating anti-Rh and anti-A and anti-B.

Incomplete anti-Rh antibody could be found in cord blood of infants with rythroblastosis more frequently than agglutinating anti-Rh, suggesting the ncomplete anti-Rh antibody is able to pass through placenta more readily than gglutinating antibody. The phenomenon of blocking antibodies was first ob-erved by Coca and Kelly in 1921 in rabbit anti-Pfeiffer bacillus serum.

In the third method the Rh antibody becomes manifest when macro-nolecular diluents are used: human serum or plasma, human or bovine lbumin (20–30 per cent), 10 per cent gelatin, acacia, polyvinyl alcohol, ectin, polyvinyl pyrrolidone (PVP), dextran and other non-protein ubstances. Human serum and bovine albumin are the best diluents. Various optimum combinations have been recommended: a mixture of ne part of 25 per cent bovine albumin and 3 parts of normal plasma; uman serum as diluent for the antibody containing serum and 30 per ent bovine albumin for the red cell suspension.

Various names have been suggested for this type of antibody: con-lutinin or glutinin or monovalent antibody (Wiener), hyperimmune ntibody (Diamond). The term serum-albumin agglutinins is widely sed. This method of Rh antibody detection is more sensitive than the revious two. In the so-called Diamond-Abelson test whole blood is ixed with Rh testing serum on a slide and read after a few minutes ncubation at about 40°C. The principle of this test employing high oncentration of red cells, a potent Rh anti-serum, and a slide is ap-licable not only to testing for the Rh factor but also to testing for Rh ntibodies, especially in pretransfusion tests.

In the fourth technic the red blood cells are coated with the agglutinin ut as in the blocking test clumping does not take place. The detection f the antibody is based on its reaction with an antiglobulin immune erum produced in rabbits by injection of human serum or globulin. The globulin of the Rh antibody which coats the red cells acts as antigen n the test.

The procedure is known as the Coombs test, but actually it was developed by Moreschi in 1908. He produced a goat anti-rabbit red cell serum which did not agglutinate rabbit cells. By using the serum of a rabbit immunized with normal goat serum, he was able to demonstrate agglutination.

The technic of the Coombs test involves: mixing known Rh-positive red cells with a serum in which Rh antibodies are being sought. The cells are then freed of serum by repeated washing with saline. To a drop of the washed red cells a drop of the antiglobulin serum is added. Clumping means a positive test. This is the indirect Coombs test or developing test. It is very sensitive and frequently permits detection of antibodies which cannot be demonstrated by the other technics.

In the direct Coombs test red cells already sensitized in vivo are tested by mixing with antiglobulin serum. It is being done with the blood of the newborn to detect sensitization, especially where there is no clinical evidence of erythroblastosis. The Coombs test is non-specific in the sense that any antibody capable of coating red cells will react with antiglobulin serum. It is used in the diagnosis of acquired hemolytic anemia for detection of auto-antibodies.

The fifth and most recent modification is based on increased sensitivity of red blood cells following exposure to enzymes (trypsin, papain, ficin). Enzyme treated red cells react readily with incomplete antibodies.

All these tests make it possible to detect Rh antibodies in close to 100 per cent of sensitized persons.

HEMAGGLUTINATION TECHNICS

Blood grouping is done by carrying out hemagglutination reactions in mixtures of:

1. Red cells of unknown blood group with typing serums of known groups (determination of the blood group factor or agglutinogen).
2. Serum of unknown group with red cells of known groups (determination of iso-agglutinins).

Hemagglutination is a special form of the phenomenon of agglutination, namely the clumping of red blood cells by the antibody (hemagglutinin) of the serum. These tests are simple and depend on the mixing of small amounts of serum containing the iso-agglutinins anti-A and anti-B, with similar amounts of suspensions of red blood cells. The tests are run at room temperature, on a slide, or preferably in a test tube. Figure 6.2 shows how the results of these tests determine the presence of the blood group factors.

A complete blood grouping includes testing for the blood group factor

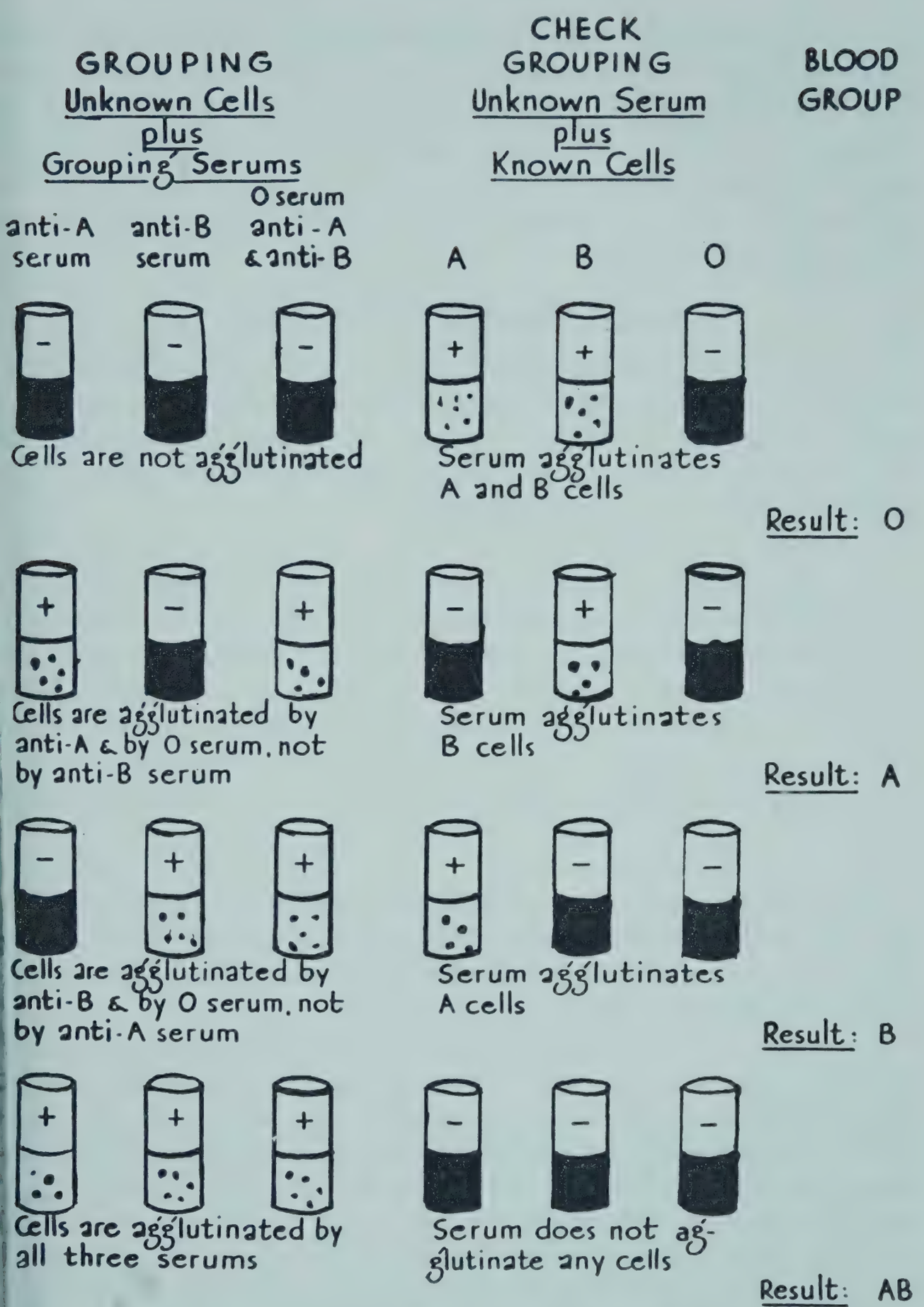


FIG. 6.2. Blood grouping tests. Note: *Results of grouping and check grouping must agree. Otherwise test is invalid.* Exception: In early infancy agglutinins in serum may be absent. (Reprinted from: Davidsohn, I.: Blood groups. Chicago U. School Quart. 16: 6, Autumn, 1954).

in the red cells with potent serums containing iso-agglutinins (blood grouping), and testing for isoagglutinins in the serum with red cells of known blood group composition (check grouping). As controls, serum of group O, which contains anti-A and anti-B, is used, and in the second part of the test, red blood cells of group O are used, which are not agglutinated by anti-A or anti-B. Such control tests offer the best safeguard against errors.

Factors Involved in Hemagglutination

1. *Sensitiveness of the agglutinogen in red cells to the agglutinating action of serum.* It varies with age, being lower in infancy and early childhood, gaining in strength with advancing age, reaching the peak around the age of twenty and declining slowly in old age. It varies in different persons for reasons unknown.

2. *Density of the suspension of red cells.*

The agglutination reaction is a two-phase phenomenon. The first phase is the union between the cell and the agglutinins, the second the formation of aggregates or clumps. If cell suspension is too heavy the agglutinins may be bound but clumping may not occur. If cell suspension is too light, agglutination may be difficult to observe. A 1 to 2 per cent suspension was found empirically to be most suitable.

3. *Quantitative relations between the cell suspension and the agglutinating serum.*

4. *Titer of agglutinins in serum.*

High titer of serum may overcome the low sensitivity of the red cells, but only up to a certain degree. In a mixture of serum of low titer and of cells of low sensitivity clumping may not occur, and the true group of the red cells may not be recognized.

The titer of iso-agglutinins varies with age. They are as a rule absent at birth. Those present at birth have been transmitted from the mother through the placenta and are eliminated during the first weeks of life. The iso-agglutinins appear slowly and gradually during the first months of life. Their titer continues to rise and reaches a peak during late adolescence; it remains at this level until about the age of fifty and then declines gradually and reaches eventually a low level in persons of advanced age. The rise as well as the drop in the titer of agglutinins is much more pronounced than is the change in the sensitiveness of the red cells. Hirszfeld suggested that both are a part of so-called constitutional serologic maturation, a manifestation of physiologic growth, analogous to other maturation phenomena.

Variations of titers of iso-agglutinins have also been observed in certain diseases. They were found by Davidsohn to be low in chronic leukemias, high in serum disease, and are low or high in many persons without any known cause.

5. *Temperature.*

Anti-A and anti-B agglutinins react characteristically within a definite range of temperature (thermal range). As a rule the reaction is more pronounced at lower temperatures and decreases in intensity as the temperature rises. On the other hand, the specificity is more pronounced at higher temperatures. The characteristic thermal range of hemagglutination reactions must be known and considered for proper interpretation of results.

6. *Mechanical agitation.*

It favors agglutination, hence the acceleration and accentuation of clumping by centrifugation.

7. *Surface tension.*

Large surface favors false agglutination such as rouleaux formation and may lead to errors, therefore rouleaux formation is less pronounced in test tubes.

Reagents:

I. Typing Serums:

Serums of groups A, B and O are needed.

A good typing serum must have the following properties:

1. It should have a high titer.
2. It should be free of cold agglutinins.
3. It should be free of so-called irregular agglutinins.
4. It should not form rouleaux when mixed with red cells.
5. It should be clear, of normal color, free of cells or any other particles, not hemolyzed, icteric or chylous.
6. It should be free of complement.

Preparation of Typing Serums. Aseptic technic must be observed, because bacterial contamination, as shown by Davidsohn and Toharsky, may make serum panagglutinating (bacteriogenic hemagglutination). Blood drawn from the vein of a young healthy person of known blood group and meeting the previously enumerated requirements is satisfactory for typing serum.

Determination of Titer. Suitable dilutions of serum in saline are prepared.

One to two per cent suspensions of red cells of groups A and B are prepared using blood of from 3 to 5 persons for each, in order to eliminate variations due to differences in agglutinating ability.

In titrating serum of group B it is desirable to have known cells of subgroups A_1 and A_2.

If the titer is found satisfactory, the serum is distributed in vials which are sealed.

Serum of group O is prepared similarly.

It is preferable not to add preservatives. Serum prepared under strictly aseptic precautions and kept in the icebox may retain its specificity and potency for many months. Frozen dried (lyophilized) typing serum keeps indefinitely.

The titer should be checked about once each month with cells of known groups, including cells of subgroup A_2 for anti-A serum. It is advisable to number each batch of serum and to keep a record of its titer and the names of the donors. Whenever possible the same donors known to have serums of high titers should be used. As a precautionary measure Rosenthal has suggested distinctive coloring of serum: a mixture of neutral acriflavine and of basic fuchsin is added to serum of group A, and brilliant green is added to serum of group B. Serum O may be used without artificial coloring.

Use of commercial typing serums. Good typing serums are commercially available. They must comply with stringent requirements of the National Institute of Health.

Patients treated with horse serum as source of high titered typing serums. Such patients, especially when they develop serum disease, have high titers of isoagglutinins, and their serum can be used as source of potent blood group testing serum.

Potent testing serums can also be produced by immunizing volunteers with appropriate antigens.

II. Standard Red Blood Cells:

For typing of serum, red cells of group A and B are needed. Fresh 1 to 2 per cent suspensions must be prepared daily. It is a good practice to prepare every morning two test tubes of suspensions of each blood group by adding a drop of blood of each group from a puncture in the ear lobe or finger to 1 cc. of saline. This gives a suspension of the desired concentration. It is advisable to type as many persons of the hospital personnel as possible and so to provide a source of donors of red cells of known blood groups.

One tube of the cell suspensions is used during the day and is kept in the icebox when not in use. It is discarded at the end of the day and the other tube which was in the icebox during the day is used during the night. Panagglutinability of red cells due to bacterial contamination may develop in eight hours if the cells are kept at room temperature. In a laboratory where many typings are done the tubes with cell suspensions may be kept outside of the icebox a good part of the day. The precaution of preparing two tubes with cell suspensions and keeping one of them in the icebox during the day for use during the second twelve hours will reduce greatly the likelihood of panagglutination. Another reason for preparing fresh cell suspensions every twenty-four

hours is the tendency of cells suspended in saline to become less agglutinable due to loss of sensitiveness.

Clotted blood is much less liable to lose its sensitiveness and to become panagglutinable in case of contamination. To avoid the trouble of having to obtain fresh blood every day, it is practicable to place a few drops of blood into a number of small test tubes without preservative or anticoagulant and to keep the stoppered tubes with the clotted blood in the icebox. Such blood keeps well for about a week. Whenever necessary suspensions can be prepared from the clots.

III. Blood of Person Whose Group Is to Be Determined:

A. Red Blood Cells:

A drop of blood from a skin puncture in the ear lobe or finger (preferably the former) added to 1 cc. of saline provides the cell suspension. It must be kept in mind that in anemic individuals more than one drop is required to give the same concentration as one drop of a normal person. It goes without saying that the skin and the puncturing instrument should be sterilized with alcohol or a similar antiseptic solution. The skin must be dry before the puncture and the first drop should be wiped off with sterile gauze.

B. Serum:

If there is an objection to entering the vein, as in the case of patients or donors whose veins have to be saved for blood transfusions, enough blood can be obtained from a skin puncture for the cell suspension and for the serum. In infants enough blood can be obtained from the heel after producing hyperemia by applying a warm wet dressing and compressing the blood vessels above the ankle. The need for serum of the donor for a test for syphilis should be kept in mind.

Technic of Blood Grouping Tests

There is a large number of methods of blood grouping employed in different places with many variants and with modifications of variants. Some of the modifications are purposeful; many embody what their authors considered useful short cuts (which seems to have been the main reason for most modifications). For some it is difficult to find a reasonable justification.

An analysis of the most widely used methods permits us to reduce them to two large groups: the slide technics and the test tube technics.

I. *Slide Technics* (see Fig. 6.3):

A. Open Slide Method:

In the slide methods a drop of the cell suspension and a drop of typing serum are placed on a slide, mixed, stirred, tilted back and forth and

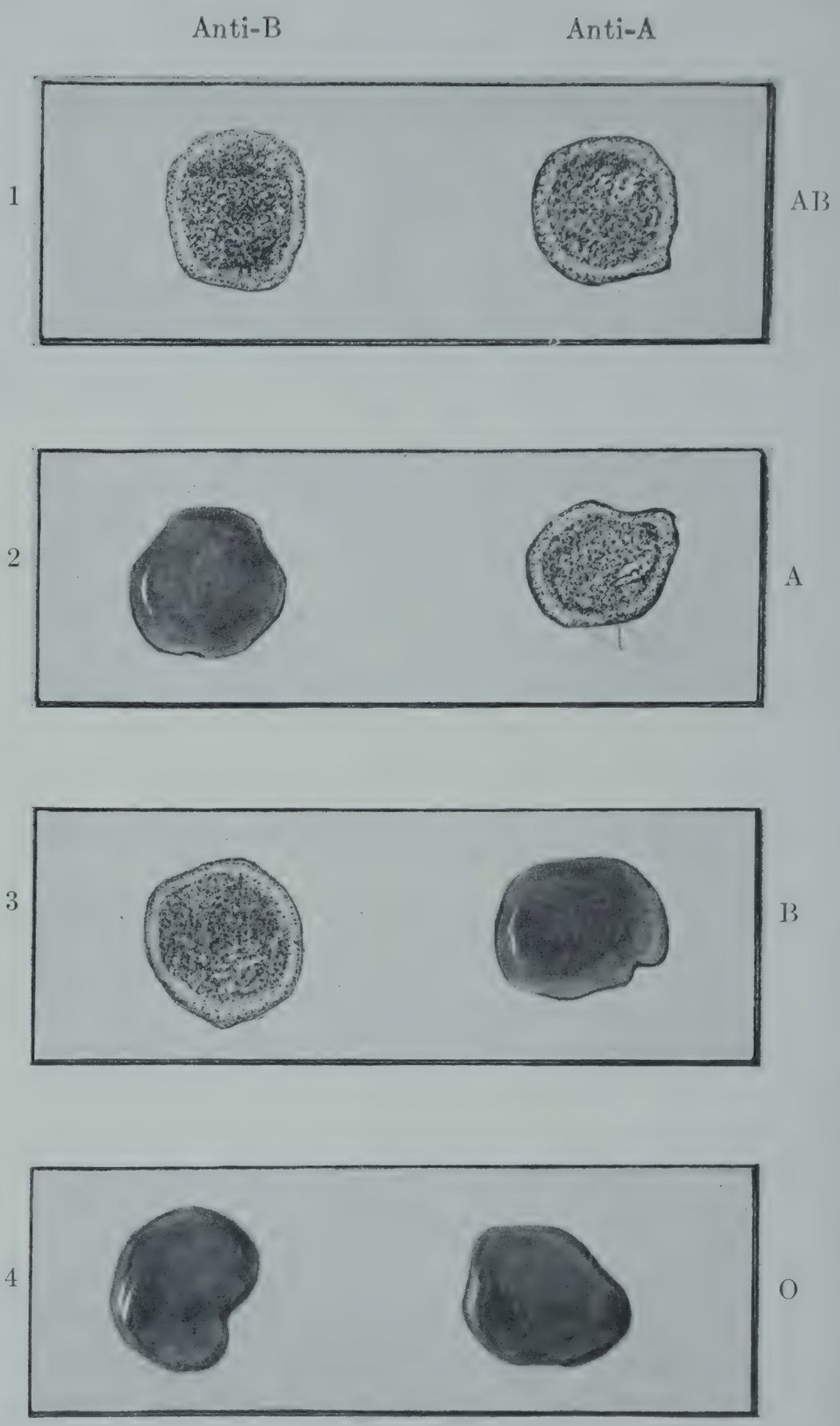


FIG. 6.3. Illustrating the four possible reactions that take place when typing blood. Assuming that anti-B serum is in the left row and anti-A serum in the right row the addition of unknown cells may result in:

1. Agglutination by both indicating that blood is Group AB.
2. Agglutination by anti-A serum showing that the blood is Group A.
3. Agglutination by anti-B serum showing that the blood is Group B.
4. Agglutination by neither, showing that the blood is Group O.

watched for agglutination. In some the mixture remains uncovered until agglutination becomes manifest or until a certain set time has elapsed when in the absence of agglutination the result is read as negative. This is the so-called open slide method. Here as in most of the other slide methods some of the modifications recommend checking seemingly negative results with the microscope before a definitely negative report is issued. Other descriptions fail to include a recommendation of checking negative readings with the microscope.

B. Closed Modification of Open Slide Method:

In a modification of the open slide method the mixtures on the slides which fail to show agglutination after several minutes of tilting back and forth (the recommended time intervals vary) are stirred and covered with cover slides and are read after certain intervals. This could be called the closed modification of the open slide method.

C. Closed Slide Method:

In this modification the mixtures on the slides are stirred and immediately covered with cover slides and read after a certain time with frequent shaking.

D. Hanging Drop Method:

A widely used modification of the closed method is the so-called hanging drop method. Here small drops of the serums and of the cell suspensions are placed on cover slides, usually with wire loops, stirred, and the slides are turned drop down over the excavation of concave microslides which are rimmed with rings of vaseline or mineral oil.

In all these methods frequent stirring and tilting is very important.

The usual procedure is to place the serums of group A on the left half of the slide and serum B on the right side.

Some recommend using serum of group O as a control, in which case a separate slide is used.

Each of these technics can also be used for typing of the unknown serum with standard cells of known groups and for the crossmatching tests in which the serum of the recipient is mixed with the cells of the donor and the serum of the donor with the cells of the recipient. For the latter test it is customary to use a slide marked with the recipients' initials in the center and the symbols PS DC (patient's serum–donor's cells) along the left margin, and DS PC (donor's serum–patient's cells) along the right to indicate the contents of the serum-cells mixtures.

II. *Test Tube Technics:*

A. The Sedimentation Technic:

One drop of saline, of serum and of the cell suspension are placed in

test tubes (inside diameter 7 mm.) and left standing for one hour at room temperature. If clumping is not seen with the naked eye at the end of the hour a drop is transferred to a slide and viewed with a microscope. Controls with known cells of groups A, B, and O are included.

B. The Centrifuge Method:

The set-up is the same as in the sedimentation technic, but after standing for 5 minutes the tubes are centrifuged for about 3 minutes at about 2,000 r.p.m. The tubes are placed in a rack and shaken till controls with cells of group O are evenly suspended.

Critical Analysis

The slide methods favor pseudo-agglutination (rouleaux formation) due to the large surface. The open slide method favors evaporation, especially in hot weather. The hanging drop method is the most economical due to the small amounts of reagents required, but it is technically a little more difficult than the others. One must watch that the wire loop used for transferring of serum and cells is not too hot. During the tilting of the slide the suspensions have a tendency to run into the vaseline ring at the margin. The concentration of the cell suspension in the droplet that is picked up with the wire loop is not infrequently below that in the test tube.

The test tube methods decrease the danger of pseudo-agglutination. The disadvantage of the sedimentation method is that it requires an hour before reading can be done. It is the method of choice when a large number of typings has to be done, and when speed is not a factor. The centrifuge method combines the advantages of the test tube technic with speed and with ease of reading results. I found it the most practical in my personal experience.

Other methods have been published and have ardent adherents. Some claim certain advantages, mainly of speed, but most of them introduce additional sources of error. As will be shown later, there are many sources of error inherent in any technic of blood grouping, even in the best, therefore introduction of additional possibilities of error for the sake of speed is to be discouraged, especially as the centrifuge method satisfies all reasonable demands for speed.

An error in blood grouping is fraught with greater danger than in any laboratory procedure. Here are some of the reasons justifying such a statement. In most other tests the clinician has an opportunity to check the laboratory report with the clinical findings in the patient. When the white blood count or a blood sugar determination is high or

when the serologic test for syphilis is positive the clinician must not accept it if it does not agree with the findings in the patient; he does not have to base his treatment, be it medical or surgical, on the laboratory result without questioning. He may repeat the test or he may repeat the examination of the patient and find an explanation for the discrepancy without jeopardizing the patient's health. Not so in blood grouping. Here the test is done as a preliminary to a transfusion. There is no relation between the blood group and the physical condition of a person. Hence the clinician has no means of checking the results. He is entirely dependent on the laboratory. If the result of the blood grouping or crossmatching test is incorrect, the error may become apparent too late to be corrected. There is no simple way of taking out the incompatible blood once it has been introduced into the patient's circulation. That is why accuracy, so important in all laboratory tests, is even more important in blood grouping and crossmatching tests. All possible checks should be included which may prevent errors and which may call attention to an error if it has crept in. This is the reason for emphasis on accuracy, on prevention of errors and on their detection. The importance of speed in blood grouping and crossmatching tests is obvious. They are carried out in life-saving emergencies more often than any other laboratory test. However, the need for speed can be met in any well organized laboratory by having a tray with all necessary reagents ready at all times and by having a blood grouping team so that one or more technicians can drop whatever task they are doing at the moment and work together to turn out the result in the shortest possible time without sacrificing accuracy. The trouble with some of the short-cut methods is that while they have something to recommend them in emergencies when time is at a premium, they are used only too often when there is not the slightest indication for excessive speed. It must always be remembered that excessive speed invites errors. The centrifugation technic meets all requirements for speed without sacrificing accuracy.

Although preference for the centrifuge method has already been expressed, many of the other methods have been tested, and it appears that most of them are good in the hands of experienced and careful workers. There are a few points which are essential for every method. The use of typing serum of group O is a valuable check because it clumps cells of all groups except its own. The typing of the serum with standard cells of groups A and B is absolutely essential and should not be omitted. Its value will, I hope, be brought out convincingly in

the discussion of sources of error. It is probably the most important means of avoiding mistakes. The additional time needed for it is brief and a very small price to pay for the greatly increased accuracy. The 1:2 dilution of typing serums is important, because pseudo-agglutination (rouleaux formation) is less frequent.

In the typing of unknown serum with standard cells of known groups, the serum is not diluted, because its strength is not known. In case of unclear results, especially in the presence of rouleaux formation, this part of the test may have to be repeated with diluted serum. However, that is rarely necessary because this is the second half of the test, and its results can be readily checked with the results of the first half.

ERRORS IN GROUPING TESTS

Sources of Error

Errors are due to technical factors or to anomalies of blood. They may be responsible for false positive or false negative results. Familiarity with them and strict adherence to the technic described here will reduce their incidence.

Causes of False Negative Reactions

1. *Typing serum of low titer.* This source of error may be caused by the failure to select typing serums of desired titer or by deterioration of originally suitable serum. The latter is especially likely to occur if the serum is kept outside ot the refrigerator. Inclusion of suitable controls and frequent testing of titer will eliminate this source of error.

False negative reactions due to low titer of iso-agglutinins may occur in typing of unknown serum with known cells and in crossmatching tests.

2. *Too heavy suspensions of red cells.* Hemagglutination requires proper quantitative relations between the red cells and the titer of agglutinins. If this relation is disturbed, for instance, if there is insufficient agglutinin or too many red cells, the antibody is bound but clumping may not become manifest.

3. *Low agglutinability of red cells* may lead to false negative results, especially when the titer of the typing serum is low. It was stated previously that the red cells are less agglutinable in infancy, childhood and old age, and that factor A is less agglutinable in subgroups A_2, A_3, and A_2B. Since stored blood came into use, loss of sensitiveness of red cells due to storage must be taken into account. As it is customary to group blood prior to storing it, the loss of sensitivity may only affect

results of crossmatching tests by the absence in certain instances of agglutination between the serum of the recipient and the cells of the donor.

There are in the literature many references to false negative results which led to the use of incompatible donors. In most of them donors were thought to be of group O, while they were actually of group A, subgroup A_2. Some recipients or donors were thought to be of group B while actually they were of group AB, most likely of subgroup A_2B. Such falsely grouped recipients were given incompatible blood or such donors were the source of incompatible blood with disastrous results in either case. In some of the reported cases crossmatching tests prior to the transfusion and rechecks of the blood grouping and of the crossmatching tests after the transfusions failed to disclose the incompatibility.

The reason crossmatching tests fail to reveal incompatibility is the same which led to the false negative blood grouping result in the first place: weak typing serum was responsible for the latter, for example, failure to recognize a weakly reacting A_2, and the serum of the patient with a low titer of iso-agglutinins was responsible for the absence of agglutination in the crossmatching test. The recheck of the grouping after the transfusion reaction would show the same result unless a stronger anti-A typing serum was used. In the repeated crossmatching test after the transfusion reaction the serum of the recipient would be more likely not to agglutinate the weakly agglutinating cells of the donor, because the previously weak agglutinins have been further reduced by the injected incompatible blood.

In some instances recheck with stronger anti-A typing serum and repetition of the tests after an interval demonstrated incompatibility, which had been overlooked previously.

4. *Lessening of agglutinability of red cells*, for example, due to the use of cell suspensions older than from 12 to 24 hours. Red cells suspended in saline show progressive loss of agglutinability.

5. *Hemolysis in grouping tests due to use of uninactivated typing serum.* Errors due to hemolysis are more likely to occur in the slide technics, than in the test tube technics, where hemolysis is detected easier, because the pink color of the supernatant fluid is readily apparent.

Hemolysis is especially likely to be seen in grouping of serum, because this reagent is not inactivated. Awareness of this possibility and correlation of the result with the grouping of the red cells will eliminate hemolysis as a source of errors. For the same reason hemoly-

sis is likely to occur in crossmatching tests, especially when a donor of group O is tested for a recipient of a different group. Here too, familiarity with the phenomenon will protect against misinterpretation.

It has been stated occasionally that inactivation of typing serum may be omitted if the serum is kept in storage for some time prior to its use for typing. Although this recommendation is theoretically sound, inactivation for 30 minutes at 56°C. is the better procedure. The amount of complement in human serum is sometimes considerable, and its deterioration in the icebox may be slow. Serums from five to six weeks old and older which have retained their hemolytic potency have been observed.

Iso-hemolysins or hemolysins in serum capable of laking red cells of the same group that are clumped by the serum are present in about 30 per cent of serums. Hemolysins are usually present only in serums with high titers of iso-agglutinins, and therefore pretransfusion tests do not as a rule require tests for hemolysins. The correlation between their titers and the fact that both are always removed together argue in favor of identity of iso-agglutinins and iso-hemolysin. Iso-hemolysis in blood grouping tests can be prevented by inactivation of typing serums. Familiarity with iso-hemolysis will help to avoid errors in crossmatching tests. Decrease of the number of red cells in the slide technics and discoloration of the supernatant fluid in the test tube technics are the hints to suggest hemolysins. Inactivation of serum for 30 minutes at 56°C. will eliminate lysis by destruction of complement and make agglutination more pronounced.

6. *Haste.* Time is one of the most important factors in the agglutination phenomenon. The time needed for agglutination to become manifest is as a rule inversely proportionate to the titer of agglutinins in the serum, to the sensitivity of the red cells and to the mechanical agitation of the serum-cell mixture.

Sometimes it may take up to one hour for the reaction to develop, especially if the mechanical factor of centrifugation is not employed. Only too often does hurry lead to a premature decision that there is no agglutination and to a false negative result.

Surgeons and others ordering blood grouping tests should be aware of the role of the time factor in these tests. On the other hand, use of centrifugation will permit shortening the time to a minimum.

7. *Failure to check negative naked eye readings with the microscope.* It would seem unnecessary to emphasize the need of checking with the microscope of macroscopically negative results. However, one does

still come across statements that macroscopic readings can be accepted without microscopic verification. Anyone with experience will agree that such statements are fallacious. Agglutination reactions vary in intensity from strong to very feeble. The latter may be below the threshold of visibility by the human eye. It is by no means certain that there is a direct relation between the strength of the agglutination reaction in the test tube or on the slide and the severity of the subsequent transfusion reaction in the human body.

Weak clumping indiscernible with the naked eye is especially liable to occur if the cell suspension is too heavy.

Causes of False Positive Reactions

1. *Pseudo-agglutination or rouleaux formation* is due to formation in serum cell mixtures of structures resembling rolls of coins (Fig. 6.4). This is undoubtedly the most frequent type of pseudo-agglutination but there are other forms of it, for example, clumps of red cells enmeshed by bacteria in heavily contaminated serums. Rouleaux formation is directly proportional to acceleration of the sedimentation rate of erythrocytes and to the level of proteins in blood serum especially of fibrinogen and globulin. Rouleaux formation is diagnostically significant for conditions associated with hyperproteinemia, for example, multiple myeloma, Kala azar, and lymphogranuloma inguinale. Rouleaux formation may simulate agglutination. Examination with the high power of the microscope will reveal the characteristic morphology; agitation and dilution of the serum with one or two parts of saline tends to cause the disappearance of the rolls while true agglutination is made more pronounced by the former and not noticeably affected by the latter.

2. *Cold agglutination due to the presence of so-called cold agglutinins.* Cold agglutinins are true agglutinins, cold agglutination is a true antigen-antibody reaction, as contrasted with pseudo-agglutination, the most common form of which is rouleaux formation. Cold agglutinins can be removed by absorption, whereas in pseudo-agglutination treatment of serum with red cells does not alter the ability of the serum to form rouleaux.

Cold agglutinins may cause errors in grouping tests, but proper technic of preparation of typing serum will free it of this interfering antibody. They may lead to errors in crossmatching tests, especially in the test between the serum of the recipient and the cells of the donor. They are found more frequently in the blood of patients with infectious diseases. Occasionally one finds cold agglutinins in the serum of pa-

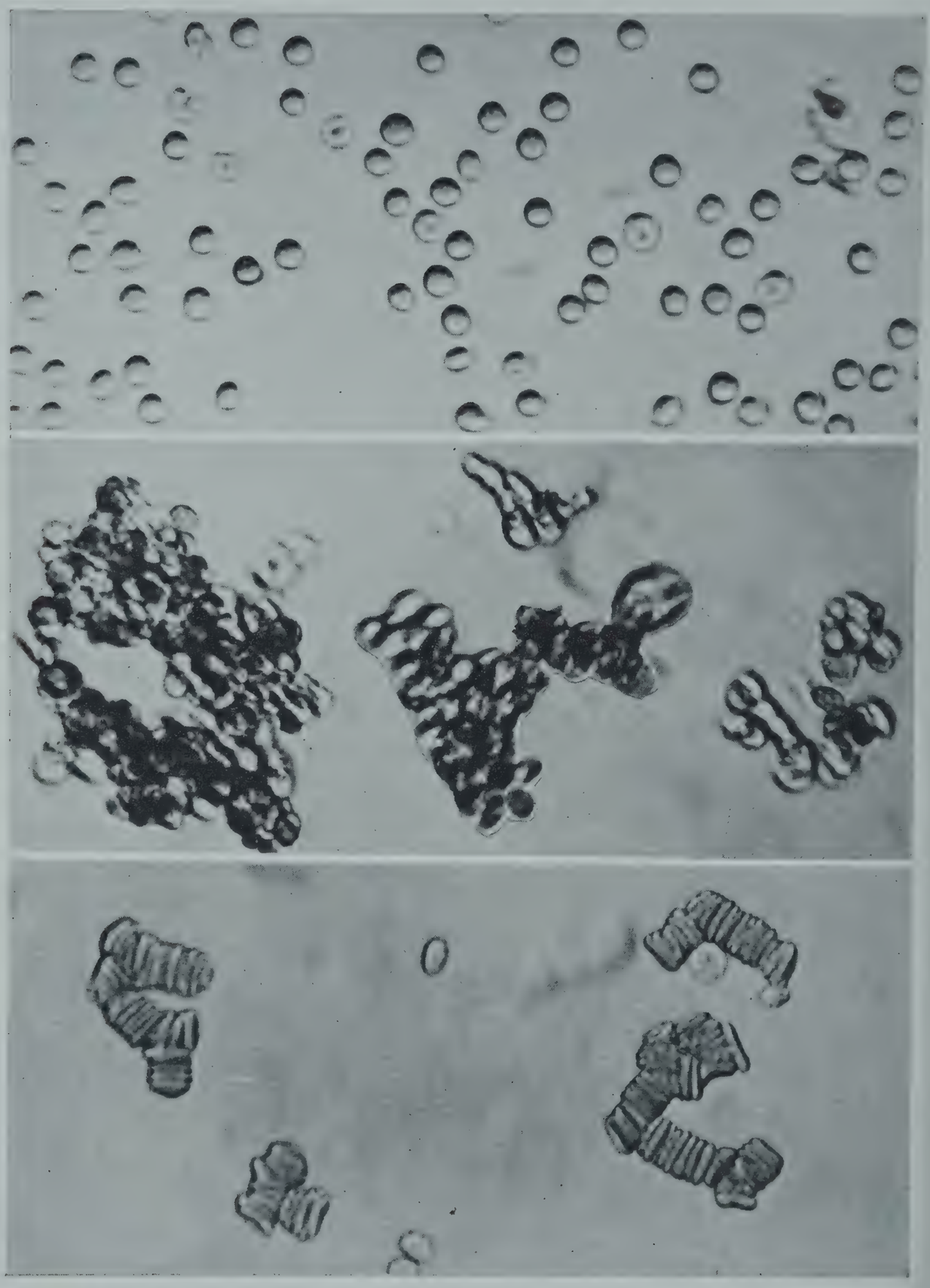


FIG. 6.4. Photomicrograph showing (top) compatible set-up, (center) true agglutination, and (bottom) rouleaux formation.

tients who had received repeated blood transfusions before and who did not show them during the previous tests. When an atypically reacting agglutinin is found in the course of a blood grouping or crossmatching test, setting up a control with cells of group O and with the cells of the

person whose serum is tested, will disclose the nature of the reaction. Placing the slides or test tubes in an incubator or in a water bath at 38°C. will promptly cause disappearance of the clumps and prove the true nature of the reaction. The clumps will reappear at lower temperatures. The consensus of authorities is that so-called cold agglutinins are not responsible for blood transfusion reactions and that agglutination in pretransfusion tests proved to be due to cold agglutinins is no indication against the use of the donor.

Cold agglutinins and hemolysins have been found in acquired hemolytic anemia.

A special form of cold agglutinins known as an auto-agglutinin is found occasionally in patients with diseases of the liver and of the blood vessels, in trypanosomiasis and in other diseases. There are instances of this type of agglutinins in individuals free of any evidence of disease. They may be responsible for errors in crossmatching tests. Auto-agglutinins react at icebox and room temperatures with red cells of all groups including the patient's own cells. The latter are clumped even when suspended in saline or in blood counting fluids; therefore, their presence is frequently detected when a blood count is done. Cells washed in saline, and then resuspended, remain unclumped. Agglutination disappears as a rule when the test is done at body temperature, but instances of auto-agglutination at 38°C. have been reported. Patients with auto-agglutinins may be given blood from suitable donors as long as crossmatching tests at body temperature show no incompatibility.

The phenomenon of autoimmunization and autoantibodies in relation to disease will be discussed elsewhere (see p. 358).

3. *Bacteriogenic hemagglutination* due to action of certain bacteria on serum, plasma or red cells. This is an occasional source of errors.

General Precautions

False positive and negative, as well as conflicting results may be due to carelessness. False results due to improper labeling of reagents or to improper recording of results are too varied to permit classification. Certain precautions should be observed: The test tubes and slides should be labeled with the name or initials of the person tested. Each test tube should be marked before blood is put into it; and if this is not done, it certainly should be so marked before the test tube is put out of hand. The tubes with the cell suspensions and clotted blood or serum of every person should be arranged in a definite order and placed in the test tube rack.

Distinctive coloring of typing serums was mentioned previously.

A very important factor in preventing clerical errors is careful recording of results in a record book. In it there should be place for recording reactions of typing serums with patient's cells and of patient's serum with cells of known groups, in addition to the usual recording of the interpretation of results. This method of recording helps to call attention to discrepancies, particularly in the hands of less experienced workers.

Strictly enforced routine in these matters is a protection against errors and permits the technician to concentrate on the work. Especially important is this in emergencies, when time counts and when tension is a disturbing factor.

The cells and serums of those who have been grouped or crossmatched should be kept in the icebox in stoppered test tubes for several days. This permits checking results if blood transfusions are followed by reactions.

Formerly, many errors could be traced to the use of conflicting nomenclature. This source of error has been gradually eliminated by the use of the so-called Landsteiner or International Classification, which employs the letters A, B and O, instead of Roman numerals.

Prevention of Errors in Blood Grouping

Adherence to the nine rules listed in Table 6.4 will prevent most errors and explain irregularities and discrepancies. They may not appeal to those who are always on the lookout for short cuts, even if the short cut means an increase in possible errors. Unfortunately, in selecting donors for blood transfusions an error, no matter how small in statistical calculations, may amount to 100 per cent for the unlucky recipient.

Meticulous adherence to these rules has led to elimination of errors in large transfusion services.

TEST FOR SUBGROUPS OF GROUPS A AND AB

Principle:

Serum of group B contains two varieties of anti-A agglutinins:

(1) Anti-A which reacts with cells of subgroups A_1 and A_2, but stronger with the former than with the latter, and

(2) Anti-A_1, which reacts with A_1 and only very feebly with A_2.

Addition of cells of subgroups A_2 to serum B removes most of agglutinin anti-A and leaves practically all of anti-A_1. The absorbed serum will clump only cells of subgroup A_1.

Test for Subgroups:

Technic is the same as described for blood grouping. Known cells A_1 and A_2 must be included as controls with each test.

TABLE 6.4

Prevention of Errors in Blood Grouping

1. Blood grouping is only half done by typing *cells with serums* of known groups.
   Typing unknown *serum with cells* of known groups completes the job and eliminates most sources of error.
   Occasionally even this may be insufficient in:
   New born and infants without iso-agglutinins.
2. Use inactivated typing serums (inactivated at 56°C. for 30 minutes) of *high titer* complying with requirements of the National Institute of Health.
   Strong anti-A typing serums are especially important to detect weak A-factor in subgroups A_2, A_3, A_1B, A_2B.
3. *Typing serums* must be *free of cold agglutinins.*
4. *Check typing serums* frequently with cells of known groups.
5. Use only *fresh cell suspensions.*
6. Concentration of *cell suspension* must be one to two *per cent.*
7. *Rouleaux formation* is best overcome by *dilution* of serum 1:3. *Agitation* makes true agglutination more distinct, rouleaux formation less distinct.
8. *Good technic.*
   Slide technic and test tube technic are equally good in hands of experienced workers.
9. *Avoid hurry.*
   Reliable typing cannot be done in a hurry. Test tube method with centrifugation is the fastest method and very reliable.

Rh TYPING

Anti-Rh Serums

Good serums can be obtained from mothers of babies with erythroblastosis and from immunized donors.

The frequency of the detectable anti-Rh agglutinins in the blood of mothers of babies with erythroblastosis varies from 50 to 95 per cent according to different writers. It increases with the number of pregnancies. The number with antibody titers suitable for use as testing serums is of course smaller. The titers, as shown by Davidsohn, are as a rule higher in blood samples obtained from 8 to 14 days after delivery than earlier. The titer may persist for considerable periods. In one case Rh agglutinins were detected six years after the birth of a child with erythroblastosis in a woman who had not received any transfusions

and who had not been pregnant in the meantime. Her first child was normal and had no history of jaundice or anemia at birth. The persistence of the antibody is, as a rule, proportionate to the initial titer.

The serums vary qualitatively and quantitatively. The most frequent variety (called anti-Rh_0 or anti-D) reacts with about 85 per cent of blood specimens. It is produced most frequently by persons of genotype cde/cde. It may also be made by genotypes Cde/cde and cdE/cde. There are occasional anti-D serums which contain two types of antibodies, anti-D + anti-D^u. D^u is a third antigen allelomorphic to D and d. It is identical with Wiener's intermediate gene. The anti-D does not agglutinate D^u red cells. Anti-D + anti-D^u agglutinates them.

Less frequent is an antiserum which reacts with 70 per cent of blood specimens collected at random. It is the anti-rh′ (anti-C) serum and is usually produced by persons of type rh (cde/cde) when immunized by red cells rh′ (C) and also by such persons immunized by red cells containing rh′ (C) and Rh_0 (D). The anti-rh′ (anti-C) antibody is usually found together with anti-Rh_0 (anti-D). Least frequent is an anti-serum which reacts with 30 per cent of blood samples collected at random. This so-called anti-rh″ (anti-E) is usually made by persons of genotype CDe/CDe or CDe/cde. Still more frequently this antibody is found together with anti-Rh_0 (anti-D) in persons of type rh (cde/cde).

Other Anti-Rh Serums

1. Anti-C^w. It was first found by Callender and Race in 1946. It is produced by immunization with C^w, which is a third antigen allelomorphic to C and c.
2. Anti-C + C^w. Most serums with anti-rh′ (anti-C) are really anti-C + C^w. The two cannot be separated by absorption. It is probable that immunization with C by itself produces first anti-C, but as immunization progresses, that slowly also anti-C^w is produced. In one case it was possible to observe that development.
3. Anti-hr′ (anti-c). It was first described by Levine and associates in 1941. It is made by persons with homozygous rh′ (C), for example, CDe/CDe, when immunized with hr′ (c).
4. Anti-hr″ (anti-e). It was discovered by Mourant in 1945.

Rh TECHNICS

It is outside of the scope of this chapter to discuss details of technic. Only general principles will be presented, as needed for the understanding of clinical problems and for the interpretation of the results of laboratory tests.

Test tubes or slides can be used. With potent commercial serums available, which meet the requirements of the National Institute of Health, technics employing slides are perfectly satisfactory. However,

technics employing test tubes have the advantage, according to some writers, that they make it possible to have the tests checked by another worker because the time limitations are not as stringent as in the slide technics.

With the exception of the anti-Hr_0 (anti-d) serum, all the other five anti-Rh serums, anti-Rh_0 (anti-D); anti-rh′ (anti-C); anti-rh″ (anti-E); anti-hr′ (anti-c); and anti-hr″ (anti-e), are available. They are available as saline agglutinating serums and as serum albumin agglutinating serums.

Most of the serums used nowadays are from immunized volunteer donors, or from mothers of babies with erythroblastosis. Commercial serums have to meet the requirements of the National Institute of Health and are, as a rule, reliable.

In using commercial test serums two important rules must be observed:

1. Follow exactly the directions of the manufacturer.
2. Test at least one Rh-positive and Rh-negative control each time you test an unknown blood. There must be no deviation from this rule. It increases the expense of testing but it is the most reliable means of obtaining trustworthy results. It is deplorable that the directions of some manufacturers refer to the use of controls as "desirable." The controls are not just "desirable," but absolutely essential. No result, positive or negative, can be accepted without them. In a large number of errors discovered in the course of examining specimens sent for checking, the failure to use controls was the cause most frequently found responsible.

The use of known Rh-positive and Rh-negative red cells for controls is, of course, essential in all tests, also when noncommercial serums are used.

The unknown red cells have to be prepared as fresh as possible. They can be obtained from a puncture of an earlobe or a fingertip. One drop of blood to 1 cc. of saline makes approximately a 2 per cent suspension. This, however, is only a rough measure. In anemic patients, more than one drop of blood may be needed.

The suspension can be prepared from a blood clot. This has the further advantage that clotted blood preserves its reactivity for agglutination tests much better than whole blood containing an anticoagulant. Cells suspended in saline must be used within a few hours.

The tests in tubes are done by adding to a drop of the test serum, a drop of the red cell suspension. This is incubated for varying periods of

time, depending upon the instructions, usually at least 30 minutes, at 37°C. Then the test is read by gently rotating the test tube and watching for agglutination first with the naked eye and if it is negative then the test tubes are centrifuged for a few minutes at from 500 to 1000 r.p.m. In general, satisfactory instructions are enclosed with each vial of serum obtained commercially.

The important thing to remember is that the Rh-negative control must be negative, the Rh-positive control positive, otherwise the test is void.

Centrifugation helps to accelerate the test.

The majority of anti-Rh serums contains so-called serum albumin agglutinins which react only when a macro-molecular protein is used as diluent.

Sources of error in tests for the Rh factor:

Clotted blood keeps well in the refrigerator for about seven days. Citrated blood keeps almost equally well. Suspensions of cells in saline must be tested within twelve hours and sometimes even this period is too long. Keeping outside of the refrigerator, especially in warm weather, shortens the period during which blood gives reliable results. Handling in transport with the attending continuous trauma adds another harmful factor.

Slightly hemolyzed blood must be tested with great caution. It tends to give false negative results. Strongly hemolyzed blood should not be tested.

Contaminated blood is unsatisfactory and likely to give false results.

When reading the results, roll or tilt tube gently to stir the sediment. Shaking, even gently, should be avoided. It may obliterate weak clumping and make a weak positive test read negatively.

Citrate in cell suspensions must be replaced by saline. Cells must be washed for the preparation of suspensions.

Incubation at body temperature gives best results. With potent serums good results may be obtained at room temperature.

Results are not reliable unless confirmed by positive and negative controls.

For mailing purposes clotted blood should be used.

In general, routine tests need to be concerned only with testing for the Rh_0 (D) factor. It is more appropriate to speak of Rh_0-positive (D-positive) and Rh_0-negative (D-negative) persons instead of Rh-positive and Rh-negative. When recipients of blood transfusions are tested it is sufficient to determine whether they are Rh_0-positive (D-

positive) or Rh_0-negative (D-negative), and when found to be Rh_0-negative (D-negative) they should be given Rh_0-negative (D-negative) blood. According to some, the selection of donors can follow the same principle with the understanding that persons with rh′ (C) and rh″ (E) blood are too rare and these antigens, as a rule, too weak to require Rh typing of donors. However, there are those who claim that in view of the danger of sensitization of an Rh-negative recipient by rh′ (C), or rh″ (E) blood, only true Rh-negative (rh, cde/cde) donors should be used for Rh-negative recipients. Tests for D^u must be included to eliminate D^u positive donors.

Tests for Rh Antibodies

The technics differ, depending on the methods used. Saline is the diluent and the suspending medium in tests for saline (complete) agglutinins. Macromolecular diluents (human serum, bovine albumin, PVP, dextran, etc.), antihuman globulin rabbit immune serum (Coombs test), and/or proteolytic enzymes (trypsin, papain, ficin) are the ingredients of the various tests for incomplete antibodies.

Test tubes or slides can be used, depending on the worker's preference.

One needs several Rh-positive bloods, preferably representing the various types of Rh. As many of these as possible are desirable, especially if one wishes to determine the exact type of the Rh antibody.

Again, for controls one must use Rh-negative cells in addition to patient's own cells.

The serum has to be inactivated for 30 minutes at 56°C.

There are various sources of error and pitfalls which only those especially trained in Rh technics can avoid and recognize.

Detailed standardization of serums with Rh antibodies should be done only in special laboratories.

Test for Blocking Antibodies

Principle:

Antibody combines with Rh-positive cells, but clumping or any other visible reaction does not occur. Subsequent addition of serum with potent anti-Rh agglutinins will fail to produce clumping of the red cells, because the latter are "blocked." Hence the term "blocking antibody."

Procedure:

A. Same procedure as for determination of anti-Rh agglutinins.

B. If results are negative, add to the negative tubes one drop of potent anti-Rh serum, diluted to a titer found suitable for Rh testing. The

serum must be of a so-called anti-Rh_0 (anti-D) variety. Reincubate for 45 minutes to one hour. Read as described previously.

Interpretation of results:

1. Clumping present in the tubes to which anti-Rh serum was added: blocking antibodies absent.
2. Clumping absent or weak: blocking antibodies present.

Open Slide Test for Rh Antibodies (Diamond-Abelson)

Procedure:

One drop of unknown serum and two drops of whole (citrated) O Rh-positive blood are mixed on slide which is placed and gently tilted on a glass plate over a 25-watt bulb.

A similar setup using O Rh-negative blood as control.

It is preferable to use known O Rh_1 (CDe) and O Rh_2 (cDE) blood. Rh-positive blood shows clumping in a few seconds to about three minutes. The reaction is accelerated and the reading facilitated by gently tilting the slide on a piece of ground glass over an electric bulb at a temperature of about 38–45°C.

Source of error is rouleaux formation. A little practice will help to distinguish this form of pseudo-agglutination from true agglutination.

This test has the advantage of being the simplest and one of the more sensitive of all tests for Rh antibodies. It is especially valuable as a screening test for Rh sensitization and as a pretransfusion test between recipient's serum and donor's blood, but does not differentiate between agglutinating and blocking Rh antibodies.

Coombs Test

Principle:

Coombs' test employs a rabbit antihuman globulin immune serum to detect Rh antibodies as well as to make reactions with weak antibodies more pronounced. The red cells are covered with human antibody globulin which reacts with the rabbit antiglobulin immune serum.

This antiglobulin test is used (a) for detection of antibodies in serum (indirect Coombs test) and (b) for detection of antibody already attached to red cells, i.e. for detection of sensitization (direct Coombs test).

(*a*) *Indirect Coombs test.*

Procedure:

First Stage:

Place two drops of the serum (inactivated for 20 minutes at 56°C.)

in a 75 x 7 mm. test tube. Add two drops of a 2 per cent suspension of Rh-positive cells. As controls set up tubes with O Rh-negative cells. Incubate at 37°C. for 30 minutes. Withdraw a small sample with a pipet and examine under a microscope. Agglutination in the test suspension and absence in the control establish the presence of anti-Rh agglutinins. Absence of agglutination may mean that the serum contains no agglutinins, the presence of agglutinins too weak to be detected, or the presence of "incomplete or blocking" antibodies. The second stage of the test may answer the question.

Second Stage:

Wash cell suspension three times with saline, then add 2 drops of saline. Add an equal volume of rabbit antihuman globulin serum. Incubate for 30 minutes at 37°C. In the presence of antibodies agglutination will become manifest.

(*b*) *Test for Detection of Sensitization of Red Cells of Infants Suspected of Having Fetal Erythroblastosis (Direct Coombs Test)*

Procedure:

To 2 drops of a 2 per cent suspension of the infant's washed red cells add 2 drops of the rabbit antihuman globulin serum. Incubate at 37°C. for 30 minutes.

Control: rabbit serum plus cells of same group as the infants, but from nonsensitized adult. Agglutination of the infant's red cells proves sensitization with Rh-antibody. The control must remain unclumped, otherwise the test is void.

The same tests are also employed in the diagnosis of acquired hemolytic anemia.

Indications for Tests for the Rh Factor

1. It is desirable that every person who needs a blood transfusion even for the first time, have his Rh type determined and if found to be Rh-negative, be given Rh-negative blood, whenever possible.

Whenever an Rh-negative person is given Rh-positive blood, he is being handicapped in case of need of transfusions in the future. He may thus become sensitized and exposed to transfusion reactions in the future.

It is possible and likely that even a first transfusion of Rh-positive blood to an Rh-negative patient may be less beneficial than Rh-negative blood would be. Transfused compatible blood remains in the recipient's circulation for from 100 to 120 days or about as long as his own blood. (This refers only to red blood cells.) According to Molli-

son Rh-positive cells have not been found in the circulation of Rh-negative recipients at the end of 60 days. The reason for the more rapid disappearance of the Rh-positive blood is that after an interval, which varies in different persons, Rh antibodies may develop and the previously compatible Rh-positive cells behave as if they were incompatible and their survival time is shortened. Thus the value of the transfusion is definitely lessened.

An Rh-negative woman who is given Rh-positive blood is being doubly handicapped. She is being placed at a disadvantage in case of need of future transfusions, and her future children are being further handicapped if she marries an Rh-positive man. Sensitization of an Rh-negative woman by transfusion of Rh-positive blood accelerates and reinforces Rh sensitization during pregnancy and makes fetal erythroblastosis in the offspring more likely, even in the first baby, which is otherwise usually not affected by the disease. This precaution applies to female recipients regardless of age, in view of the fact that sensitization to Rh once established seems to be a lifelong state.

2. A person in need of a blood transfusion and who has received transfusions before or who is likely to need transfusions in the future should have his Rh type determined and, if found to be Rh-negative, be given Rh-negative blood.

3. A patient in need of a transfusion, who has received transfusions before and who had a reaction, even a mild one, following a previous transfusion, not necessarily the last one, should have his Rh factor determined, and if found to be Rh-negative, should be given Rh-negative blood.

It has been observed that reactions due to sensitization of Rh-negative recipients with Rh-positive blood are at first mild, but when overlooked are followed by severe or even fatal reactions.

4. A pregnant woman or a woman in childbirth or in puerperium or after recent childbirth or abortion in need of a transfusion should have her blood Rh tested.

Giving Rh-positive blood to such an Rh-negative recipient is absolutely contraindicated. Rh sensitization in pregnancy of Rh-negative wives of Rh-positive men is as a rule much more intensive and of longer duration than sensitization resulting from transfusing Rh-negative recipients with Rh-positive blood.

5. It is a good rule never to use a husband as a donor for his wife, because she is exposed during pregnancies to sensitization by his blood factors, some known and still more probably unknown. For the same

reason, the husband's blood relatives and the woman's previously born children should not be used as donors.

6. A newborn child showing some of the manifestations of fetal erythroblastosis (anemia, jaundice at birth or soon afterwards, or edema) should have the Rh type determined.

7. Members of hospital staffs, including nurses, internes and professional donors should have their Rh type determined to have Rh-negative donors of all groups available, especially for obstetrical emergencies.

APPLICATION OF BLOOD GROUPS TO BLOOD TRANSFUSIONS

Pretransfusion Tests

Selection of donors, for blood transfusion, is based on the principle that individuals of the same blood group as the recipient be selected, and that their blood be matched with recipient's blood in two tests. These so-called crossmatching tests introduced by Hektoen in 1907 are:

1. The so-called "major crossmatch," in which the serum of the recipient is mixed with the red blood cells of the donor. Any clumping, no matter how feeble, eliminates the donor.

Recently, highly sensitive crossmatching tests have been introduced. One method employs macromolecular mediums, such as bovine albumin or human serum for suspension of the red cells. In another method, the indirect Coombs test is used for matching the serum of the recipient with the red cells of the donor. These technics permit detection of incompatibilities not demonstrable with older methods.

2. The so-called "minor crossmatch," in which the serum of the donor is tested with the red blood cells of the recipient. Here, too, no clumping should be present, except in the case of the so-called "universal donor." The record of this procedure, together with the record of the blood groupings of the recipient and donor, is entered in the hospital record and becomes a permanent part of it.

The reason for the designation of these two tests as "major" and "minor" is that the first one establishes a possible reaction between the antibodies in the whole volume of the plasma of the recipient, with the relatively small volume of transfused red cells. In the case of a reaction, the danger is much greater than the reaction depicted in the second test, where the possibility of an agglutination between the rapidly diluted, small amount of the donor's plasma with the larger amount of the recipient's red blood cells is considered. Theoretically, it is assumed that the second reaction would be less dangerous, due to the effect of

dilution on the injected antibody. It is this reasoning which gave rise to the conception of the so-called "universal donor" of group O. In this group, under usual circumstances, no agglutinable substances are present in the red cells. Thus, it was assumed that the iso-agglutinins, anti-A and anti-B, present in the donors' plasma, would become rapidly diluted in the plasma of the recipient, thereby eliminating any serious consequences. This reasoning still applies in most instances, but there are occasional individuals of group O whose iso-agglutinin titer is exceptionally high. In these cases, the dilution resulting from the transfusion of one pint of blood to a person with about ten to twelve pints of blood, is not sufficient to eliminate the possibility of a reaction, resulting from the combination of these antibodies with the recipient's red cells. Actually, transfusion reactions, even fatal ones, have been observed under these circumstances. Therefore, the use of the so-called "universal donor" has been restricted to donors with low titers of isoagglutinins.

It has been recommended that the titer of these antibodies in the plasma of group O donors, for recipients of different blood groups, be determined by means of a rough titration which shows if the antibodies against A or B are present in a titer, set arbitrarily at 1:100 to 1:200. An individual with a titer below this level is considered as meeting the requirements of a so-called "universal donor." However, more recently, it was found that in addition to natural iso-agglutinins, some individuals have so-called "immune" iso-agglutinins. These immune iso-agglutinins are much more dangerous than natural ones, even if present in very low titers. Some fatal transfusion reactions have actually been traced to the presence of these. The main reasons why "immune" iso-agglutinins are more dangerous than the natural ones, are their enhanced reactivity at body temperature and their resistance to neutralization.

Witebsky described a method of detecting these immune iso-agglutinins, by adding to such a serum a certain amount of soluble blood group specific substances. They neutralize the natural iso-agglutinins only; they do not neutralize the iso-agglutinins of immune origin, or do it much less efficiently. These immune iso-agglutinins can be detected by using diluents other than saline. A test was recently described by Grove-Rasmussen which makes it possible to detect immune antibodies in a simple cross-matching test.

Three-tenths milliliter of the group-specific substances is added to 0.1 ml. of the donor's serum. Two drops of the mixture are placed in each of 2 small test tubes. Two drops of a cell suspension of group A_1 are added to one tube and

similarly group B cells to the other. After incubation at 37°C. for 1 hour, agglutination serves to exclude the intended donor. In the absence of clumping, the supernatant is removed and a Coombs test is done on the cells. If it is negative, the donor is safe. If positive, the blood should not be used, regardless of the titer.

There are situations in which the use of a universal donor is the lesser of two evils. This is the case in emergencies when lack of time does not permit waiting for the selection of a donor of the same group, especially if the requirement is that he be Rh-negative, as in obstetrical emergencies.

The objections against the use of so-called universal donors hold also against the use of incompatible donors for recipients of group AB or so-called universal recipients. The absence of iso-agglutinins in their serum is supposed to be a sufficient safeguard because the reaction between the serum of the recipient and the cells of the donor is eliminated. However, the presence of high titers of iso-agglutinins in the serum of a donor may be objectionable. A similar procedure as was outlined for the elimination of dangerous universal donors should be used here.

So-called universal recipients may have irregular iso-agglutinins, a potential source of transfusion reactions. A person of subgroup A_1B may have anti-O irregular iso-agglutinins which would bar use of blood of group O, and of subgroup A_2 and A_2B, if the agglutinin reacts at body temperature. Irregular agglutinin anti-A_1 is found in about 30 per cent of subgroup A_2B, thus making administration of blood of subgroups A_1 and A_1B potentially dangerous. Various factors (infectious diseases, repeated transfusions) may cause increase in titer of irregular iso-agglutinins.

The selection of suitable donors is not limited to the principle of blood group compatibility. Other tests are done in order to eliminate the possibility of the transmission of certain diseases, including syphilis, malaria, and especially, homologous serum jaundice. The donor also has to meet certain physical requirements in order to protect him against complications resulting from the removal of an amount of blood that might be excessive for his physical condition. These tests include a thorough medical history, physical examination, tests for hemoglobin, etc. For the protection of recipients and donors, standards have been set up by the National Institutes of Health and by the American Association of Blood Banks.

Pretransfusion Tests for Rh Compatibility

Just as pretransfusion tests for the usual blood groups include selection of a donor of the identical group and crossmatching tests, so do Rh

pretransfusion tests for an Rh-negative recipient include selection of an Rh-negative donor and a crossmatching test.

Pretransfusion Tests for Infants and Children

Donors of blood for infants and children must be selected with the same care as for adults, including blood grouping and crossmatching tests. The occasionally encountered statements that crossmatching is not necessary because infants do not have iso-agglutinins is not valid, because agglutinins appear in infants at various ages, sometimes rather suddenly. Furthermore, it is likely that absence of agglutinins in circulation does not assure safety of a transfusion. Antibodies may be attached to tissues. Finally, the agglutinins in the transfused plasma may harm the infant's blood.

Iso-agglutinins are as a rule absent in the newborn. This makes it impossible to carry out the important, so-called major part, of the crossmatching test (between the recipient's serum and the donor's cells). This can be overcome by crossmatching the mother's serum with the donor's cells. The result will apply to the baby. A blood incompatible with the serum of the mother will also be incompatible with the infant, and vice versa.

TRANSFUSION REACTIONS RESULTING FROM BLOOD GROUP INCOMPATIBILITY

Transfusion of blood may be followed by one of several untoward reactions. They include: pyrogenic or febrile reactions, allergic reactions, heart failure, embolic, and hemolytic reactions. Only the latter will be discussed here, because they are most commonly the result of errors in pretransfusion tests and because they may present diagnostic problems requiring laboratory tests for their solution.

In most instances, the clinical picture of hemolytic transfusion reactions is characteristic and permits a diagnosis without great difficulty. However, there are at least three reasons which justify preoccupation with this subject: (1) there are cases in which the clinical manifestations are obscured by the underlying disease; (2) the characteristic clinical picture of hemolytic transfusion reaction is not always fully developed; and (3) complications of diseases following a transfusion may be interpreted as caused by transfusions. Fortunately, there are laboratory tests which can help in establishing the correct diagnosis, provided that they are used properly and at the proper time.

There are situations in which the aid of the laboratory is essential for the correct diagnosis of hemolytic transfusion reactions.

(1) Asymptomatic hemolytic transfusion reaction. Clinical symptoms such as typical anginal precordial pain, severe lumbar distress, chill followed by drop in blood pressure, are frequently the first indications of a hemolytic process. However, the absence of these signs and symptoms does not exclude a hemolytic transfusion reaction. Hemolytic reactions have occurred in spite of absence of clinical manifestations, and with definite proof available that incompatible blood was administered.

(2) Hemolytic transfusion reaction may be simulated by underlying disease, and

(3) Hemolytic transfusion reaction may be obscured by the underlying disease.

Here belong: progressive renal failure, which may be a part of the transfusion reaction, but which may also be a part of the underlying disease or a complication of a surgical operation; acute liver insufficiency with jaundice; acute anemia; hemoglobinuric nephrosis due to causes other than transfusion hemolysis and resulting from shock or hemorrhage; progressive azotemia resulting from massive gastro-intestinal hemorrhage; jaundice following massive pulmonary infarction, etc. The changes in the blood of patients with hemolytic transfusion reactions which are responsible for the various clinical manifestations described in the preceding sentence are: intravascular hemolysis followed by hemoglobinemia, hemoglobinuria, bilirubinemia, and bilirubinuria; rise in non-protein nitrogen and its fractions, indicative of renal failure secondary to hemoglobinuric nephrosis. Intravascular hemolysis demonstrable by these tests in transfusion reactions is in most instances the result of destruction of injected red cells by the isoantibody of the recipient, or sometimes the result of destruction of the red cells of the recipient by the antibody of the injected blood. Our discussion will deal mainly with recognition of hemolytic transfusion reactions of this nature. Intravascular hemolysis may also occur when the transfused blood contains a large amount of free hemoglobin, or excessively fragile cells (inadequately preserved or over-aged blood). Renal and/or hepatic failures may be caused by various factors. These are some of the reasons why a transfusion may be blamed for events it did not bring on and, vice versa, why a diagnosis of a hemolytic transfusion reaction may be missed.

In addition to the chemical tests already mentioned, other tests are indicated to establish or exclude the possibility of serologic incompatibility as the cause of the clinical manifestations.

In Table 6.5 are listed various forms of incompatibility in the ABO blood group system. In the left half of this table, donors are listed

TABLE 6.5

Causes of Hemolytic Transfusion Reactions due to Blood Group Incompatibility

I. ABO incompatibility

Donor		Recipient		Result: Agglutination and/or Hemolysis of Red Cells of
Antigen	Antibodies	Antigen	Antibodies	
A		B	anti-A	Donor
AB		B	anti-A	Donor
B		A	anti-B	Donor
AB		A	anti-B	Donor
A		O	anti-A, anti-B	Donor
B		O	anti-A, anti-B	Donor
AB		O	anti-A, anti-B	Donor
O	immune or "high-titer" anti-A, anti-B	A		Recipient
		B		Recipient
		AB		Recipient
A	immune or "high-titer" anti-B	AB		Recipient
B	immune or "high-titer" anti-A	AB		Recipient

whose iso-agglutinogens can react with iso-agglutinins found in the blood of the recipients shown in the corresponding horizontal column. Little need be said to explain the consequences of administering incompatible blood when the recipient has antibodies for the transfused cells. The situation is different in reactions caused by incompatible iso-antibodies in the blood of the donor. This possibility is shown in the lower half of the table where iso-agglutinins in the donor's blood can react with the iso-agglutinogens of the recipient.

The most common intragroup hemolytic reactions are caused by administration of Rh_0(D)-positive blood to Rh_0(D)-negative recipients (Table 6.6). This concerns mainly recipients previously sensitized by transfusion and/or pregnancy. Such reactions can be avoided when modern methods of crossmatching and selection of blood are observed. The top part of Table 6.6 contains the most commonly encountered situations, in which one deals with Rh-negative recipients possessing antibodies for the Rh_0(D) and/or other Rh antigens and hence ca-

TABLE 6.6

Causes of Hemolytic Transfusion Reactions Due to Blood Group Incompatibility

II. Rh incompatibilities

Donor		Recipient		Result: Agglutination and/or Hemolysis of Red Cells of
Antigen	Antibody	Antigen	Antibody	
Rh_1 (CDe)		rh (cde)	anti-Rh_0 (D)*	Donor
Rh_2 (cDE)		rh′ (Cde)	anti-Rh_0 (D)*	
Rh_1Rh_2 (CDE)		rh″ (cdE)	anti-Rh_0 (D)*	
rh′ (Cde)		rh (cde)	anti-rh′ (C)*	
rh″ (cdE)		rh (cde)	anti-rh″ (E)*	
Rh_1 (CDe)		Rh_2 (cDE)	anti-rh′ (C)†	Donor
Rh_1 (CDe)		Rh_0 (cDe)	anti-rh′ (C)	
Rh_2 (cDE)		Rh_1 (CDe)	anti-rh″ (E)†	
Rh_2 (cDE)		Rh_0 (cDe)	anti-rh″ (E)	
hr′-positive (c)		hr′-negative (CC)	anti-hr′ (c)†	
rh (cde)	anti-Rh_0	Rh_0(D)-positive		Recipient
X,Y,Z‡-positive		X,Y,Z,-negative	anti-X,Y,Z‡	Donor

* Always of immune nature.

† These reactions occur only exceptionally.

‡ X,Y,Z stand for the recently discovered blood factors Kell, Duffy, Kidd, etc., as well as the even more numerous as yet unknown antigens of the red cell.

pable of destroying transfused blood cells containing these agglutinogens. In some instances, Rh-negative blood with Rh antibodies may be transfused to Rh-positive recipients, and in this manner conditions prevail similar to those when "dangerous universal donor" blood is given to recipients possessing the corresponding agglutinogens. Sensitization of Rh_0(D)-positive recipients to one of the Rh-antigens other than Rh_0(D) is much rarer and, as a rule, more difficult to detect. Similar difficulties may arise with recipients sensitized to the hr′, Kell, or Duffy factors. These and other rarely involved, or even unknown factors (to be discussed later), are indicated by the last horizontal column of Table 6.6 referring to "X, Y, and Z" antigens.

Investigation of Transfusion Reactions

Which steps are to be taken in order to make possible a complete investigation of transfusion reactions if and when they occur? The following measures are recommended: (1) every pre-transfusion specimen must be saved for a period of at least 10 days, together with the pilot tube of the blood that was crossmatched for the recipient; (2) after administration of blood, the bottle should be returned to the blood bank for reasons to be mentioned later; (3) whenever a transfusion reaction is reported or suspected, a specimen of blood of the recipient must be obtained immediately, one part of which is placed into a tube containing an anticoagulant, while the rest is used as clotted blood for tests to be mentioned shortly; (4) a specimen of urine is obtained immediately; (5) additional specimens of urine are collected and measured as indicated.

Table 6.7 lists laboratory tests to be utilized for recognition of hemolytic transfusion reactions. The following tests are carried out immediately: (1) donor and recipient are retyped (ABO group, Rh type); (2) the crossmatching tests are repeated with pretransfusion and post-transfusion specimens, using in both instances a sensitive high protein crossmatch, as well as the indirect antiglobulin (Coombs) test. If all tests are negative but there is good reason to suspect a hemolytic transfusion reaction, a crossmatching test with enzyme-treated red cells of the donor is done; (3) the supernatant plasma of the post-transfusion specimen is examined with the naked eye and chemically for the presence of free hemoglobin; (4) a drop of the non-clotted blood is examined under the microscope for presence of small agglutinates resulting from intravascular agglutination; (5) serum bilirubin and urea nitrogen are determined on pretransfusion and post-transfusion specimens, and these tests are repeated at 24 hour intervals if a hemolytic transfusion reaction has occurred; (6) urine specimens are tested for free hemoglobin, red cells and red cell casts; (7) the few drops of blood always remaining in the bottle should be tested for ABO and Rh type in order to be sure that the blood in the pilot tube and in the bottle are identical. Another portion of the remaining blood should be used for study of possible bacterial contamination. Table 6.7 lists tests which furnish corroborative evidence and further insight into the mechanism of hemolytic transfusion reactions.

If all these tests give negative results, one can exclude a hemolytic transfusion reaction. If positive results have been obtained, the identification of the antibody responsible must be approached by the usual methods. In some instances, when a hemolytic transfusion reaction is

TABLE 6.7

Laboratory Tests in the Diagnosis of Hemolytic Transfusion Reaction

A. Tests of primary importance

Material Examined	Test	To Be Used for Detection of	Optimum Time of Examination after Transfusion	
			From	To
Pretransfusion specimen of recipient	ABO and Rh typing	Technical, or clerical error	Immediate	
Pilot tube of unit of blood transfused and remainder of blood in bottle	ABO and Rh typing	Technical, or clerical error	Immediate	
Remainder of blood in bottle	Bacteriologic study	Bacterial contamination	Immediate	
Pretransfusion specimen and pilot tube	Crossmatch	Agglutination indicative of incompatibility	Immediate	
Post-transfusion specimens of recipient:				
Whole citrated blood	Microscopic examination	Presence of clumps	Immediate	6–12 hours
Urine	Hemoglobin	Hemoglobinuria	Immediate	48 hours
Plasma, or serum	Hemoglobin	Hemoglobinemia	Immediate	48 hours
Serum	Bilirubin	Bilirubinemia	24 hours	96 hours
Blood, or serum	Urea nitrogen	Azotemia (rising urea nitrogen)	36 hours	

B. Tests of corroborative nature

Material Examined	To Be Used for Detection of	Optimum Time of Examination after Transfusion	
		From	To
Red cells of recipient	Presence of incompatible agglutinogen	Immediate	6–12 hours
Serum of recipient	Presence of incompatible agglutinin	Immediate	6–12 hours
Serum of recipient	Methemalbuminemia	6 hours	24 hours
Serum of recipient	Low isoagglutinin titer	Immediate	4–5 days
Serum of recipient	Rising isoagglutinin titer	6 days	
Urine	Albuminuria	Immediate	Variable
Urine	Biliburinuria	24 hours	4–5 days
Biopsy or autopsy: Kidneys	Hemoglobinuric nephrosis	36 hours	10 days

suspected, one must also investigate the possibility that this was due not to blood factor incompatibility, but to other causes of hemolysis such as inadequate preservation of blood (improper temperature of storage), simultaneous administration with the blood of incompatible solutions, such as glucose in water, distilled water, etc. It is, therefore, highly desirable that records of the administration of blood contain a definite statement as to the solutions administered, together, before, or after the blood passes through the same set of tubing.

Once a hemolytic transfusion reaction has occurred, follow-up tests are useful and important for management of the patient and evaluation of the effectiveness of therapy. As shown in Figure 6.5, a fairly constant chain of events takes place. Changes in serum bilirubin, bilirubinuria, azotemia and urinary output follow a predictable course, and are of great prognostic value for the ultimate fate of the patient. Hemoglobinemia and hemoglobinuria may be demonstrable within a few hours after administration of incompatible blood; peak values of this most direct evidence of intravascular hemolysis are found most frequently about 24 hours later, while 48 hours after the transfusion they can be detected only exceptionally. Jaundice and its corollary, bilirubinemia, become manifest approximately 24 to 48 hours later. Renal insuffi-

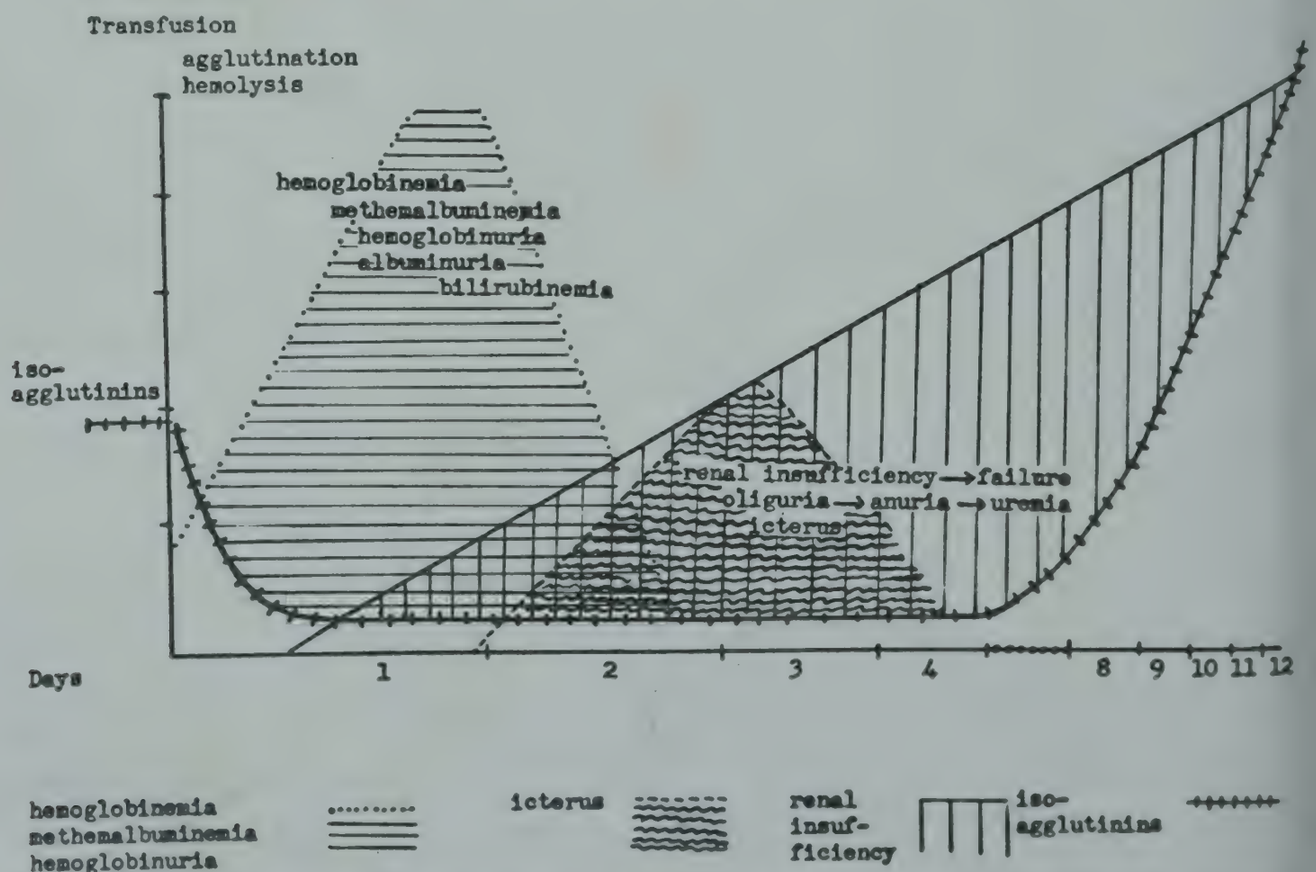


FIG. 6.5. Course of laboratory and clinical findings in hemolytic transfusion reactions.

ciency, which may culminate in anuria, with resulting accumulation of nitrogenous wastes in the blood, may become noticeable any time after the first 24 hour interval following the transfusion. It is the complication which actually decides the ultimate fate of the patient: either subsiding with recovery of the patient, or proceeding toward a fatal outcome. Changes in the titer of the iso-agglutinins responsible for the transfusion reaction are of considerable interest: immediately after administration of the blood a considerable drop in antibody titer takes place, because of removal by antigen-antibody union. Subsequently, that is within the next four to five days, the antibody rises, reaching a high titer within one to two weeks following administration of incompatible blood.

The Rh Factor in Blood Transfusions

The Rh factor aroused much interest outside of the narrow circle of those especially interested in blood groups because Wiener, Levine and others applied the knowledge promptly to problems intimately connected with medical practice.

These investigations explained for the first time so-called intragroup hemolytic transfusion reactions occurring occasionally in patients receiving carefully matched and apparently compatible blood of their own group. Such reactions have been known to occur in patients who had previously received several transfusions without reactions and in pregnant women even without a history of previous transfusions.

The previously named authors showed that these reactions occur almost exclusively in Rh-negative persons in whom previous transfusions of Rh-positive blood produced a state of sensitization to the Rh-factor. Rh-negative women may become similarly sensitized in the course of pregnancy with an Rh-positive fetus. In many instances anti-Rh agglutinins could be demonstrated, and subsequent transfusions of Rh-negative blood were well tolerated.

The reason for the failure to detect incompatibility in the pre-Rh era was that testing for the Rh factor and for anti-Rh agglutinins requires a special technic, more sensitive and delicate than tests for the usual iso-agglutinins. The iso-agglutinins anti-A and anti-B, which are used to detect the four blood groups, A, B, AB, and O, have, as a rule, a higher titer than anti-Rh agglutinins. They react within a wide range of temperature, from icebox temperature, where the titer is at its peak, declining slightly through room and body temperature, and remaining detectable, in most instances, even at temperatures close to

50°C. Anti-Rh agglutinins react, as a rule, best at body temperature, and lose their strength rapidly with changes of temperature in either direction so that at room temperature, at which the usual blood grouping and crossmatching tests are done, Rh-agglutination is frequently difficult to detect or even absent, except with serums with a high titer of agglutinins. Rare instances have been reported of anti-Rh agglutinins reacting better or exclusively at icebox temperature.

Thus, it is apparent, that the reason for the failure to discover Rh-incompatibility prior to the introduction of the special sensitive technic was due to the usual methods of doing grouping and matching tests on slides and at room temperature, while Rh agglutination requires that the tests be done in test tubes, at 37°C., and that the reaction be reinforced by fairly long incubation or by centrifugation.

The Rh Factor and Erythroblastosis Fetalis

According to Levine fetal erythroblastosis is due to damage of the blood forming and other tissues of the Rh-positive fetus by Rh antibodies coming through the placenta from the Rh-negative mother in whom they were produced by transplacental passage of Rh-positive fetal blood. This hypothesis was confirmed by: (1) statistical evidence (over 90 per cent of mothers of erythroblastotic babies are Rh-negative as compared with 15 per cent in other women); (2) birth of twins, one of them, the Rh-positive, having erythroblastosis, the other the Rh-negative, remaining well (Kariher); and (3) the birth of a healthy Rh-negative child following artificial insemination of an Rh-negative woman with semen of an Rh-negative man, after repeated births of erythroblastotic Rh-positive children.

Levine's discovery influenced the treatment of the disease. It was shown that Rh-negative blood is to be used for transfusing the babies, because Rh antibodies passively transmitted from the mothers may react with transfused Rh-positive blood. The demonstration of Rh-antibodies in mother's milk by Witebsky, Anderson, and Heide is the basis for the interdiction of nursing by the mother.

Where the mothers of erythroblastotic babies are Rh-positive, the cause of the disease has been found in differences in the types of Rh, in some cases, in A or B factor incompatibility between mother and infant in others, and finally, in incompatibility caused by new blood factors discovered only recently. Occasional cases are seen without any detectable blood group incompatibility.

An Rh-negative individual receiving transfusions of Rh-positive blood

or an Rh-negative woman pregnant with an Rh-positive fetus, may become sensitized, and this sensitization is as a rule a lifelong condition. It is claimed that about 50 per cent of Rh-negative individuals who receive an Rh-positive blood transfusion become sensitized. Sensitization during pregnancy is apparently less readily acquired because it takes more than one pregnancy, as a rule, for it to develop.

The practical application of our knowledge of the Rh factor has influenced profoundly the practice of blood transfusions. If donors are carefully selected and only homologous Rh blood is administered, then most of the unexpected transfusion reactions that used to be so troublesome in the past can be eliminated. Only homologous Rh blood should be given: Rh-negative (cde/cde) blood to Rh_0-negative (D-negative) recipients, and Rh_0-positive (D-positive) blood to Rh_0-positive (D-positive) recipients. In this way, Rh-negative (cde/cde) recipients will not receive rh′ (Cde) or rh″ (cdE) blood capable of causing sensitization, Rh-negative blood will not be used unnecessarily and will not be responsible for the rare but possible sensitization of a homozygous Rh-positive recipient. In the case of women recipients, the urgency of using only homologous Rh blood is still greater, because in addition to the handicap of developing sensitivity and being endangered in case of a future transfusion, by becoming sensitized in this way, their firstborn children may have fetal erythroblastosis, otherwise a rare occurrence. Women sensitized by transfusions may never be able to give issue to a healthy child.

It is now generally agreed that most cases of fetal erythroblastosis are due to Rh incompatibility between mother and child, usually in such a way that the mother is Rh-negative and the child Rh-positive. AB incompatibility is responsible for fetal erythroblastosis more frequently than had been assumed heretofore.

The development of Rh antibodies in the mother in response to the entrance of Rh-positive blood or of its components from the fetus into the circulation of the mother through the placenta, and the passage of such antibodies back from the mother through the placenta into the fetus, with consequent damage to the fetal blood, anoxia, damage to the liver, to the brain, etc., is the recognized pathogenesis of fetal erythroblastosis. All manifestations and pathologic changes can easily be explained in this way (Fig 6.6).

The serologic setup of Rh incompatibility favoring erythroblastosis exists in approximately 13 per cent of marriages and in about 10 per cent of all births, because it is in this 13 per cent of marriages that the husband is Rh-positive and the wife is Rh-negative, and in 10 per cent

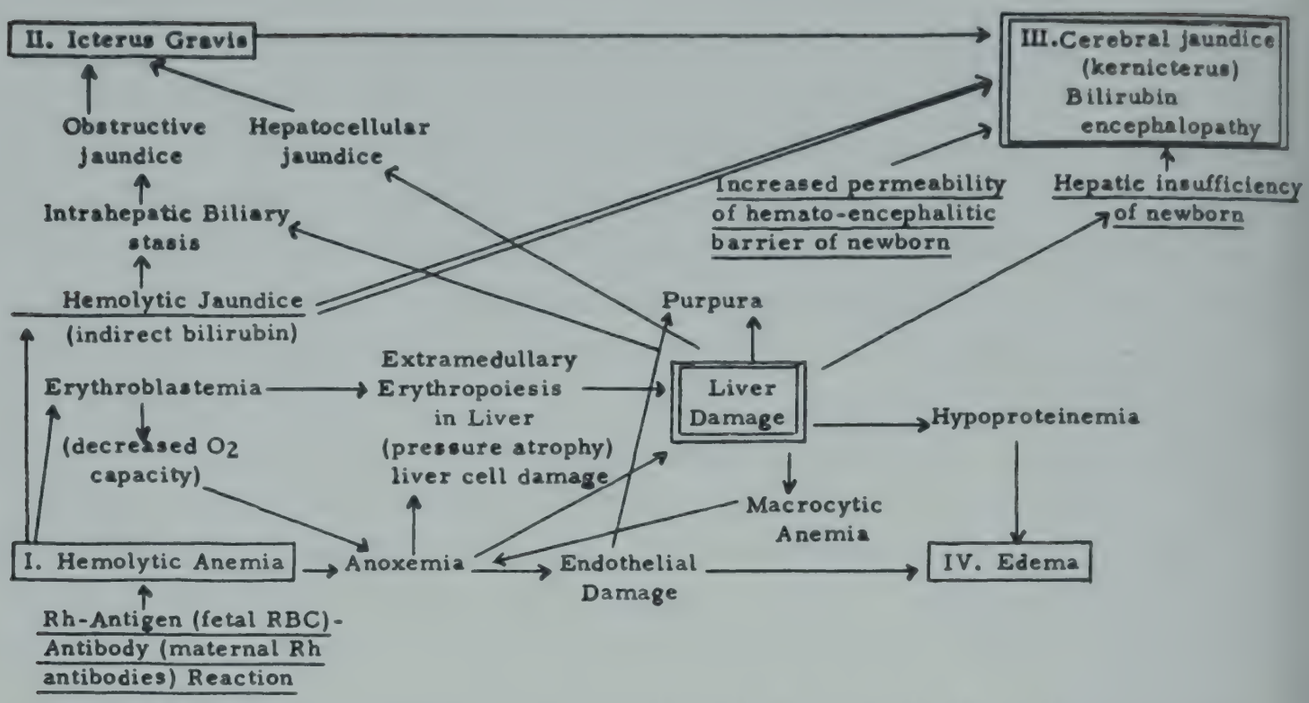


FIG. 6.6. Pathogenesis of fetal erythroblastosis and of cerebral jaundice

of births that the mother is Rh-negative and the child Rh-positive. In spite of that, the incidence of the disease is actually considerably less, about once in from 200 to 300 deliveries. The difference between the rarity of the disease and the relative frequency of the serologic setup which forms the necessary background is explained by several circumstances.

It takes, as a rule, more than one pregnancy for sufficient sensitization to develop. The antigen varies in its antibody stimulating capacity. The antibody producing capacity of women differs from person to person. Finally, the factor of permeability of the placenta for the antigen from the fetus to the mother and for the antibodies in the opposite direction is apparently a variable quality.

Fetal erythroblastosis due to Rh incompatibility is also possible in children of Rh-positive mothers and is then caused by the difference in the types of the Rh factor.

The Diagnosis of Fetal Erythroblastosis

The diagnosis can be made from clinical findings, supported by hematologic data. The pathologist can make the diagnosis from autopsy findings. Serologic findings based on the study of the Rh factor, are as important as the clinical and anatomic and sometimes even more decisive.

The serologic study may be prenatal or postnatal.

The first step of the study is finding evidence of a serologic difference, i.e., the husband has to have a different Rh type than the wife. Most

frequently, the husband is Rh_0 (D)-positive and the wife Rh-negative. Other forms of type difference occur occasionally. One must not draw too far-going conclusions from finding a serologic difference in Rh type. Serologic incompatibility is present when in addition to Rh type difference there is evidence of Rh sensitization of the mother to the husband's blood antigen in the form of Rh agglutinins. One has to consider the possibility that sensitization and Rh antibodies may be the result of a previous pregnancy, or of a previous transfusion of blood, and must not necessarily be the result of the current pregnancy. Such antibodies are called carry-over antibodies.

There was a time when we used to find Rh antibodies in mothers of babies with erythroblastosis only in a relatively small percentage. Now, with the improved methods of determination, we find them in almost 100 per cent of cases.

The absence of Rh antibodies in mothers of erythroblastotic babies, although occurring, is rare. Sometimes the titer of antibodies may be so low as to be hardly detectable at the end of pregnancy, and even immediately after childbirth, but the examination about 10 to 12 days after delivery may detect a substantial amount of them. The study of these serologic factors, i.e., Rh type differences and evidence of Rh sensitization, may help considerably, especially in cases where the differential diagnosis of fetal erythroblastosis has to be considered. A prenatal study makes it possible to anticipate and be prepared for the treatment of the newborn. For details of differential diagnosis see Table 6.8.

The postnatal diagnosis of fetal erythroblastosis is made on the basis of clinical and laboratory findings. The latter include a blood count, an Rh test of the infant's blood (finding that it is Rh-negative makes fetal erythroblastosis very unlikely), a Coombs tests on the infant's blood cells, a test for free Rh antibodies and a quantitative bilirubin test in the infant's serum.

Another form of the disease in which serologic studies are essential is so-called latent erythroblastosis, in children born with no signs of illness, in whom the disease becomes manifest sometimes in a very severe form on the second or third day of life. Early therapy immediately after birth may be decisive in saving such infants. In such cases, several laboratory tests may be of great help in selecting early suitable therapy. These tests include a blood count and hemoglobin done on the cord blood. It has been shown that blood counts and hemoglobins done on peripheral blood of newborn babies are notoriously not reliable partly because tying the umbilical cord late may give the baby

TABLE 6.8

Differential Diagnosis of Fetal Erythroblastosis in Live Born Infants

	F.E.*	C.S.	A.I.	P.I.	I.D.	I.N.	I.P.	PJK§	H.D.N.	C.M.B.D.	C.M.H.
1. Familial incidence	+++	+	−	−	−	±	+	−	−	−	−
2. Splenomegaly	+++	+++	+	±	+	+	±	−	−	−	+
3. Heptomegaly	++	++	±	±	+	−	±	−	−	+	+
4. Enlarged placenta†	±	++	−	−	−	−	−	−	−	−	−
5. Anemia	++	++	++	±	+	−	±	−	±	−	−
6. Leukocytosis	++	++	++	++	+	−	±	−	±	−	−
7. Leukemoid blood picture	++	++	++	+	+	−	−	−	±	−	−
8. Erythroblastemia‡	++	++	+	−	+	−	−	±	±	−	+
9. Severe and early jaundice	++	++	+	+	+	−	−	− (+3rd day or later)	−	−	−
10. High blood bilirubin	++	++	+	+	+	+	+	+	−	+	−
11. Indirect van den Bergh reaction	++	++	+	±	+	+	+	+	−	−	−
12. Bile in urine	++	++	+	−	+	−	±		−	+++	−
13. Purpura	±	+	±	±	±	−	−	+(lung, brain)	+++	+	−
14. Edema, general	±	+	−	−	−	−	−	−	−	−	−
15. Edema, local	±	+	−	−	±	−	−		−	−	−
16. Extramedullary hematopoiesis at term	+++	+++	±	−	+	−	−	−	−	−	+
17. Hepatic biliary stasis	+++	+++	+	−	+	−	+	−	−	+++	−
18. Liver cell damage	+++	+++	±	±	+	−	−	−	−	+++	−
19. Hemosiderosis	+++	+++	±	±	+	−	−	−	−	−	−
20. Nuclear jaundice (Kernicterus)	+	−	−	−	−	−	−	+	−	−	−
21. Positive tests for syphilis	−	+++	−	−	−	−	−	−	−	−	−
22. Father Rh+, mother Rh−, infant Rh+	++ (in 95%)	−	−	−	−	−	−	−	−	−	−
23. Rh antibodies in mother's blood	++ (in over 90%)	−	−	−	−	−	−	−	−	−	−
24. Coombs test	++	−	−	−	−	−	−		−	−	−

+++ always present; ++ rarely absent; + frequently present; ± may be present; − absent.

* Abbreviations: F.E.—Fetal erythroblastosis; C.S.—Congenital syphilis; A.I.—Antenatal infection P.I.—postnatal infection; I.D.—Inclusion disease; I.N.—Icterus neonatorum; I.P.—Icterus praecox *PJK*-PJK syndrome or sixth day disease; H.D.N.—Hemorrhagic disease of newborn; C.M.B.D.—Congenital malformation of bile ducts; C.M.H.—Congenital malformation of heart.

† The average weight of a normal placenta is about one-seventh of the total body weight.

‡ Erythroblastemia indicates the presence of immature nucleated erythrocytes in the circulation in excess of 1000 per cu. mm.

§ Prematurity-Jaundice-Kernicterus syndrome; new entity in premature babies (Aidin, Read: M Press, 226: 88–93, 1951).

Reprinted from: Davidsohn, I.: Blood Groups. Chicago. M. School Quart. 16: 9. Autumn, 1954.

an amount of blood equivalent to a blood transfusion and, vice versa, tying it early may deprive the baby of a similar amount. Hemoglobin of 14 gm. or less and a red count of 4,500,000 or less, at term, are considered as indicating anemia. In view of the fact that only Rh-positive children can have fetal erythroblastosis due to Rh incompatibility, this finding at birth favors the possibility of the disease. A Coombs test if positive, favors the diagnosis of latent fetal erythroblastosis. It proves that Rh antibodies from the mother have entered the circulation of the infant and have been deposited on the baby's Rh-positive blood. Finally, a test for bilirubin should be done. Its result may have prognostic as well as diagnostic implications.

Occasional cases of severe anemia in children several months old, of late kernicterus, etc., may also require serologic study as a help in the diagnosis. These are cases of late fetal erythroblastosis in which the disease may have been absent in the neonatal period or it may have been mild and overlooked.

In general, the fear of consequences resulting from a marriage of an Rh-positive man and an Rh-negative woman is greatly exaggerated. Figure 6.7 illustrates the reasons for this hopeful point of view. The large circle represents all women. Those who are Rh-positive need not be concerned, as a rule. Only those within the interrupted circle, who are

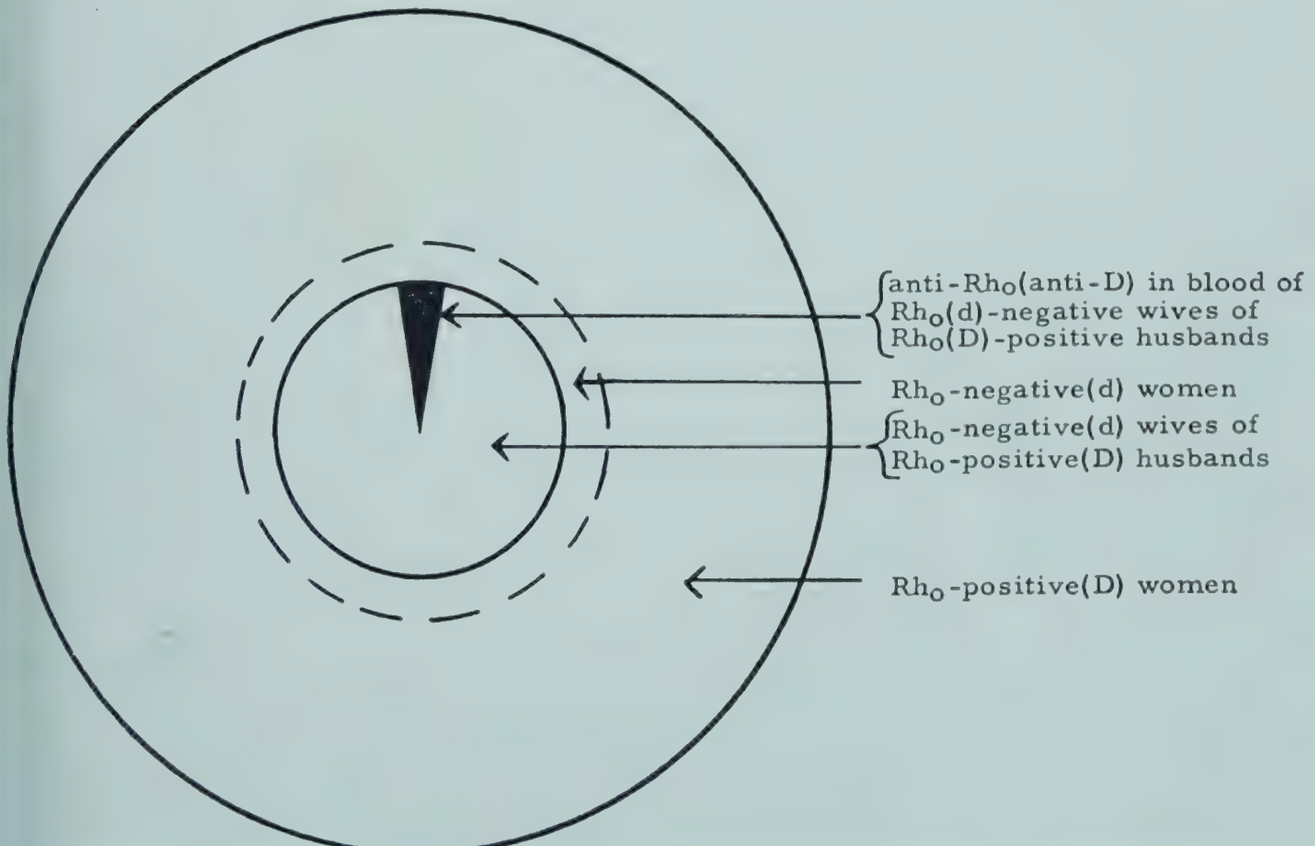


FIG. 6.7. Prenatal screening tests. (Figure designed by Dr. Kurt Stern.)

Rh-negative and again only those of them who have Rh-positive husbands, and of the latter group only those who have Rh antibodies and who are represented by the wedge, may have children affected by the disease. Even those will have, as a rule, no trouble with the first child.

The Prognostic Value of Serologic Tests

The physician is frequently confronted with the question of prognosis in fetal erythroblastosis.

The serologic examination of the mother's blood for antibodies may have a prognostic significance. Many other factors need to be considered. Much depends upon the parity of the patient. A woman who is found to be Rh-negative, whose husband is Rh-positive, and who is in her first pregnancy, has an excellent chance of having a perfectly normal first child, because first-born children are very rarely affected by the disease. Certain circumstances in the obstetrical history have a definitely aggravating significance. They include a history of abortion. Abortions have a similar though slightly less pronounced sensitizing influence as have pregnancies. Also included, of course, are stillbirths, which have even a more serious prognostic significance. Transfusions, unless it is known that they were given with homologous Rh blood, have such a similar aggravating significance. It was shown that a great many cases of erythroblastosis in first-born children could be traced to sensitizing influence of a transfusion of non-homologous Rh blood.

Twins seem to have a greater tendency to produce sensitization. It is possible that the double source of the antigen is a factor.

Another prognostically important consideration is the Rh_0 homo- or heterozygosity of the father. If he is homozygous Rh_0-positive (D-positive), i.e., he inherited the Rh_0 (D) factor from both parents, then the prognosis is much worse than otherwise, because an Rh_0 (D) heterozygous father may give issue to an Rh-negative child, whereas all children of Rh_0 (D) homozygous fathers have to be Rh_0-positive (D-positive) and hence subject to the disease and contributing to increased sensitization of the mother.

Studies of Rh antibodies during the pregnancy may be of prognostic significance.

The different types of antibodies vary considerably in their prognostic significance (Table 6.3). The prognosis in cases when so-called serum albumin antibody is present is much more serious than when saline antibodies alone are present. The so-called true blocking antibody has the most serious significance because many women who developed such

blocking antibodies during pregnancy have stillborn babies, as a rule due to fetal hydrops. It is therefore important to do tests for the various types of Rh antibodies. It is desirable to do the tests repeatedly during the pregnancy, the first time before the end of the first trimester, because antibodies found that early in pregnancy are as a rule the result of a previous pregnancy. It is obvious that the prognostic significance of antibodies is different in those cases in which they are the result of a preceding pregnancy than in cases in which they have developed in the current pregnancy. One may, for example, be disturbed by antibodies found in the latter part of pregnancy and then be surprised by the birth of a perfectly normal Rh-negative child. An examination done during the first trimester of pregnancy could have disclosed the presence of antibodies which, at that time, could not be due to the current pregnancy. Opinions differ regarding the advisability of doing these tests during the pregnancy and regarding the frequency with which they should be done. It has been recommended that in addition to the tests done during the first trimester of pregnancy, at least three more tests should be done: one some time during the fifth month of pregnancy, another during the seventh month and the third preferably immediately before delivery. In this way one may be able to get an idea about the trend of the antibody development. Experience teaches that when antibodies are absent during the pregnancy and appear for the first time four weeks or less before delivery, one can be very hopeful that the child will not be affected at all or only very mildly. The finding of antibodies for the first time about two months or less before term has also a relatively good prognostic implication. On the other hand, the finding of antibodies during the fourth or fifth months of pregnancy has a more serious implication.

Also significant is the titer of the antibodies. Here again one can draw no conclusions from a single examination. A consistently high titer, and especially a rising titer, make the prognosis serious. The value of such repeated tests is two-fold: they permit making a cautious prognosis, they warn of danger and permit making suitable arrangements prior to the delivery. See Tables 6.9, 6.10, 6.11.

Serologic Aspects of Treatment

It is generally agreed that Rh-negative blood should be used in spite of the fact that the infants are themselves Rh-positive. The reason for it is that there are Rh antibodies in the circulation and/or attached to the reticulo-endothelial tissues of the infant which react or may react

TABLE 6.9

Fetal Erythroblastosis—Prognostically Significant Points

(1) The Rh status of the pregnant woman and of her husband
(2) The homozygous or heterozygous status of the Rh-positive husband
(3) History of transfusions received by mother
(4) History of abortions
(5) Parity
(6) Past obstetrical history
(7) Tests for Rh antibodies during pregnancy
    (a) The importance of the quality of antibodies
    (b) The importance of the titer of antibodies
    (c) Measures tending to decrease sources of error in doing tests for Rh antibodies: so-called comparative tests
    (d) Importance of the state of pregnancy at which the antibodies become manifest

TABLE 6.10

Prognostic Evaluation of Prenatal Tests

	Favorable	Unfavorable
(1) Appearance of antibodies	Late	Early
(2) Type of antibodies	Complete*	Incomplete†
(3) Antibody titer	Low	High
(4) Change in titer	Absent	Rise (terminal drop)
(5) Rh type of husband	Heterozygous	Homozygous

* Antibodies producing agglutination in saline medium.

† Antibodies incapable of producing agglutination in saline medium, but producing agglutination only under special conditions (macromolecular mediums, indirect Coombs test, enzyme-treated red cells).

TABLE 6.11

Fetal Erythroblastosis—Prognostically Aggravating Factors

(1) Presence of so-called incomplete Rh antibodies (frequently referred to as blocking antibodies)
    (a) in a high titer
    (b) for longer than four weeks before term
    (c) when present repeatedly at every test
(2) In ABO homospecific pregnancies
(3) When the father is homozygous Rh-positive
(4) The baby of male sex
(5) Prematurity

In the *presence* of all these factors the baby's chance to escape fetal erythroblastosis is almost nil. As the number of positive factors decreases, the chances of escaping disease increase.

with the injected Rh-positive blood, thus contributing to the continuation of the disease and to its possible aggravation.

The treatment of choice is the so-called exchange or replacement transfusion, with one or more units of blood being exchanged, the amount depending on the severity of the jaundice. If the serum bilirubin is higher than 16 mg./100 ml., one unit is not sufficient. Furthermore, if repetition of the test for serum bilirubin shows continued high levels or an elevation, and the Coombs test continues to be positive, another replacement transfusion is indicated as soon as possible, preferably within twelve hours after the first.

Rh-negative blood of an ABO group compatible with the blood of the mother is used. Blood compatible with the mother is chosen, because the newborn has no iso-antibodies, except those received from the mother. If the baby is of a group different than O, and O blood is used, blood group specific substances are added in order to neutralize the iso-antibodies. Recent experience indicates that severe jaundice is prognostically much more serious than anemia. It has also been recognized that one must take care not to increase the blood volume of the infant excessively, because heart failure may supervene. In order to increase the volume of red cells, without increasing the blood volume, it has been found useful to remove a portion of the plasma from the blood after centrifugation or sedimentation. In this way the hematocrit of the transfused blood is raised to fifty-five or sixty. An exchange of equal volumes, with this high hematocrit blood, permits a more complete replacement of the Coombs test positive red cells with O Rh-negative blood of the donor.

When single blood transfusions are given, 10 ml. of blood per pound of body weight has been recommended. Recently there is a tendency to give resuspended Rh-negative blood, that is, blood cells without the plasma. The removal of the plasma has been justified by the assumption that adult plasma contains a factor necessary for the agglutination of red cells and its addition is supposed to favor such agglutination. According to some, clinical experience seems to support the use of such resuspended blood.

Indication for blood transfusions is the presence of clinical fetal erythroblastosis. In the case of latent fetal erythroblastosis the findings, as outlined previously, of anemia in cord blood, of a positive Rh factor in the cord blood of the newborn, and of a positive Coombs test, together with a history of erythroblastosis in a previously born infant, are an indication for treatment, even in the absence of clinical manifestations of the disease.

The most important contribution to the prevention of fetal erythroblastosis is the recognition of the relation between incompatible Rh transfusion and erythroblastosis in the first-born baby, suggesting the absolute indication for Rh homologous blood in transfusions to women regardless of age. It has also been suggested that occasionally intramuscular injections of blood given to young infants or children may mean sensitization and trouble in the future. The objection that can be raised against this possibility, in view of the small amount of blood injected intramuscularly, is countered by the fact that some individuals are apparently very prone to sensitization and that in such cases the quantity of the antigen may not be important.

AUTOANTIBODIES AND DISEASE

Here belong antibodies, mainly agglutinins, and less frequently, hemolysins, capable of reacting with the individual's own red cells. They are so-called incomplete antibodies (see p. 363), which means that they are capable of combining with red cells without a demonstrable reaction. The latter becomes manifest only in a favorable environment, provided by a suitable diluent and temperature of incubation. Red cells suspended in saline may become coated with incomplete antibodies but they do not aggregate and clump, except in the presence of macromolecular diluents such as serum-albumin, dextran, polyvinylpyrrrolidone, etc.

The underlying principles of the various improved methods of detecting autoantibodies differ from each other. Macromolecular diluents create favorable physical-chemical conditions for the reaction of incomplete antibodies. In the antiglobulin test, the antibody globulins deposited on the surface of red cells react with the antiglobulin immune serum. In a third method, red cells become more sensitive by the action of proteolytic enzymes and incomplete antibodies are made to react visibly even in saline medium.

With the help of the technics just mentioned, autoantibodies can be detected in most cases of idiopathic, and in many of symptomatic acquired hemolytic anemia. Autoantibodies react not only with the cells of the host, but, as a rule, with the cells of other persons. In some instances, autoantibodies react best at 37°C. (warm autoantibodies), in other cases, best at icebox temperature (cold autoantibodies).

In addition to autoantibodies reacting with the host's own red blood cells, evidence has been accumulating that there are antibodies capable of clumping leukocytes of all varieties under proper experimental condi-

tions (leukoagglutinins). The data thus far gathered permit a few general statements. The substance in the serum responsible for the agglutination of leukocytes has the characteristics of an antibody. It is present in the gamma globulin fraction. Both group specific and nonspecific leukoagglutinins were found in persons with leukopenia, which suggests that they are an autoantibody.

Immune antigen-antibody reactions have also been described in thrombocytopenic purpura. Antibodies clump platelets with resulting thrombocytopenia.

All this adds up to the recognition of immunologic mechanisms involved in deficiencies of all three cellular elements of blood as shown in Table 6.12.

The concept of an autoantibody as a substance produced in our own body in response to an autoantigen, a component of our own body, and reacting with and exerting a damaging influence on elements of our own tissues, has been gaining recognition gradually, and is now generally recognized. It is not surprising that the autoantibody first to be discovered was one reacting with the red blood cells. Clinical manifestations of anemia are more common and more readily noticed than those brought

TABLE 6.12

Immunohematologic Mechanisms Involved in the Pathogenesis of Diseases of the Three Components of Blood

Involved Component	Antibody	Clinical Condition
1) Erythrocytes	Cold or warm hemagglutinins auto hemolysins	Autoimmune hemolytic disease (AHD) (Acquired idiopathic or symptomatic hemolytic anemia)
(2) Leukocytes	leukoagglutinins auto leukolysins (?)	Immuno-leukopenia, idiopathic or symptomatic
(3) Platelets	thromboagglutinins auto thrombolysins	Immuno-thrombocytopenia, idiopathic or symptomatic

on by leukopenia or thrombocytopenia. There is good reason to believe that there are more conditions to which the phenomenon of an autoantigen-autoantibody reaction may be applicable than those already recognized.

Some writers have applied a good descriptive term, diseases of autoaggression, to this field of medicine dealing with damage to man's tissues by antibodies or antibody-like substances produced in his own blood.

Although the term "autoantibody" is used widely and freely, it should be kept in mind that there are those who question the propriety of this designation. They claim that there is no proof that the substance responsible for the reaction is an antibody. It could be a chemical substance, a globulin which attaches itself to the cells or structures in question without being a true antibody. They call such substances paraantibodies. This question will have to be settled by further research. In the meantime, the term "autoantibody" may be employed as a useful hypothesis.

There are also those who claim that autoantibodies are not the cause of this form of acquired hemolytic anemia, but the result of cellular or tissue destruction.

OTHER BLOOD GROUPS

In recent years various new blood group factors have been discovered. Two of the reasons for this increasing frequency of detection of new blood group factors are the more prevalent use of transfusions, due to greater availability of blood in blood banks, and the advances of surgery, which are followed by greater losses of blood and necessitate administration of larger volumes preceding, during and after operations. Another reason is the improvement of methods for the detection of incompatibilities. The result is that during the last ten years, an ever increasing number of new blood factors has been discovered, some of them responsible for transfusion reactions and some for hemolytic disease of the newborn. Usually these discoveries followed a fairly consistent pattern. First, an agglutinin was found in a serum which reacted differently from other known agglutinins. The red cells which reacted with the new agglutinin were recognized as containing the new blood factor. In this way a new agglutinable property was discovered in the red blood cells. When larger numbers of individuals were tested with this serum, it was found that some individuals reacted with the agglutinins and others did not. A statistical study showed the distribution of the new blood group, its behavior with regard to heredity, etc. Such agglutinating

serums were found as a rule in the blood of women who had been pregnant one or more times or, in patients who were given blood transfusions, and in some instances developed hemolytic transfusion reactions, and in others showed no such reactions. Occasionally the antibodies were found in individuals who had no history of pregnancy or transfusion. The names of the new blood factors have been taken from the persons in whose blood they were found.

The P System

Factor P was discovered by Landsteiner and Levine in 1927. There are two types, P-positive and P-negative. The testing serum, anti-P, may be found in the serum of man, more frequently in women, and in non-immunized horses, rabbits, pigs and cattle. The frequency of P is about 75 per cent P-positive and 25 per cent P-negative in Caucasoids. In Negroes, about 97.36 per cent were found P-positive.

The P antigen is independent of the other blood group antigens. It seems that the antigen is not fully developed at birth. It is inherited as a Mendelian dominant.

In about one-fifth of all transfusions, P-positive blood is given to P-negative people. The fact that antibodies do not develop frequently, indicates that P must be a poor antigen. It has never been found responsible for hemolytic transfusion reactions.

The Lewis Blood Groups

An antibody found in 1946 in two samples of serum led to the discovery of a new blood factor known as the Lewis factor, from the name of one of the donors of the blood. Since that time several other examples of the same blood group were found. In adults factor Le^a occurs in 21 per cent of the British population. Strangely, the factor is seen in higher numbers in infants than in adults. It is detectable in infants up to three months of age in 79 per cent, from four to six months in 73 per cent, from seven to nine months in 36 per cent, and from ten to twelve months in 29 per cent. This may be explained by the suggestion that in adults only homozygotes give the reaction, whereas in young infants the heterozygotes also give the reaction. Another very interesting phenomenon was reported when it was found that all Lewis-positives are also non-secretors of A and B, and vice versa. The Lewis system has been found responsible for hemolytic transfusion reactions in a few cases.

The Kell Blood Group

The Kell blood group was described in 1946 with the help of an antibody found in the blood of a mother of a child with fetal erythroblastosis. The anti-Kell is an incomplete antibody and is best detected with the

help of the antiglobulin (Coombs) test. The antibody seems to be fairly frequent. It has been found responsible for fetal erythroblastosis and for hemolytic transfusion reactions. The Kell factor is present in from 7 to 10 per cent of British population, who are called Kell-positive, possessing the K factor; the balance is Kell-negative and have the k factor. Those who lack the K factor possess the k factor. This was confirmed by the discovery of an antibody reacting only with the k factor. In 1949 a new blood factor was discovered designated as Cellano. It was found subsequently to be identical with k and to be present in 98.8 per cent of the American population. The k (Cellano) factor was found capable of causing fetal erythroblastosis.

The Lutheran Blood Groups

The Lutheran blood groups were discovered in 1946 in the serum of a patient suffering from lupus erythematosus disseminatus. This patient had received many transfusions before and produced a long list of various irregular antibodies (anti-c, anti-C^w and anti-Levay, about which more will be said later). The antigen was found in one of the transfused bloods and hence the name of the blood group. The Lutheran blood factor was found to be independent of ABO, MN, S, P and Rh systems, as well as of the other blood group factors discovered since. It is assumed at the present time that there are two antigens in this blood group system, Lu^a and Lu^b, and the serum which was found in the patient mentioned was designated as anti-Lu^a. The anti-Lu^b has not been found as yet, therefore, the gene Lu^b can only be recognized when Lu^a is absent. There are two phenotypes, Lu (a+) and Lu (a−).

Several examples of this antibody were found subsequently. This patient had a hemolytic transfusion reaction due to anti-Rh_0 (anti-D). Approximately 8 per cent of the British population was found to be Lu (a+) and 92 per cent Lu (a−).

The Duffy Blood Groups

The Duffy blood group was discovered in 1950. The antibody was found in the blood of a hemophiliac who received several blood transfusions during the past twenty years. The factor, Fy^a, was found in 65 per cent of the English population. It is independent of the other known blood groups just as the other blood group factors. The antibody is known as anti-Fy^a. Duffy is the name of the patient in whose serum the antibody was found. More recently, the complementary blood factor Fy^b was found with the help of the homologous antibody, anti-Fy^b.

The antibody is of the incomplete type and can be detected only with the indirect antiglobulin test and not with the albumin diluent or enzyme-treated red cells. This factor was responsible for several recorded hemolytic transfusion reactions.

The antigenicity of this factor is quite low, inasmuch as Duffy-positive blood is given to Duffy-negative patients in approximately twenty-three per cent of all transfusions, and reactions are relatively rare. Anti-Duffy natural antibodies have only been reported in one case.

The Kidd Blood Groups

This blood group system was discovered in 1951. The antibody labeled anti-Jk^a was found in a mother who had just delivered her sixth child, the first to suffer from fetal erythroblastosis. The antigen Jk^a is present in 75 per cent of white persons tested.

Family Blood Factors

In addition to the previously listed nine blood group systems, present in various percentages in the general population, blood factors present only in members of a single family or generally prevalent but absent only in members of a single family, have been described. Table 6.13 lists most of these recently discovered so-called "family" blood factors, but an ever-increasing number is being published at frequent intervals. A

TABLE 6.13

*"Private" Blood Factors**

Name	Antigen		Titer	Antibody Technic Used	Origin	Antigenic Stimulus
	+†	−‡				
Levay (1946)	3/7	350	1:20	Saline agglutinin	Immune?	Transfusion
Gr. (1946)	5/8	191	1:32	Saline agglutinin	Natural	None
Jobbins (1947)	2/4	120	1:1024	Indirect antiglobulin test	Immune	Pregnancy
Miltenberger (1951)	4/9	320	1:16–32	Indirect antiglobulin test	Immune	Pregnancy
Jay (1951).	6/8	1500§	1:8	Hemolysin	Immune	Gastric tumor(?)
(1952)	0/1	112§	1:128	Saline agglutinin	Immune?	Pregnancy?
Berrens (1952)	5/9	574	1:512	Serum-albumin, indirect antiglobulin test, enzyme-treated RBC	Immune	Transfusion and pregnancy

* Based in part on table by P. Levine (Tr. New York Acad. Sc. II, 13: 205–209, 1951).

† Number of specimens positive out of total tested in the family.

‡ Number of negative (or positive) specimens in the random population.

§ Random samples of population positive.

more detailed account of the family blood factor discovered in our laboratory, is typical of those discovered by others.

Soon after birth, a woman's second child developed typical findings of fetal erythroblastosis. Examination revealed that the mother was B, Rh-positive; the husband B, Rh-negative; and the child B, Rh-positive. The mother had an antibody, which reacted only with the blood of the husband and four other of his blood relatives. Several hundred other persons were tested and none of them had the new blood factor. As is customary in these cases, the blood factor was labeled with the family name, Berrens. Ths history revealed that the woman had been transfused with the husband's blood after the birth of their first child. This transfusion and the second pregnancy were sufficient to sensitize her and to produce the antibodies. The first pregnancy was not responsible for the sensitization because the first child was Berrens-negative.

Other such reports indicate that a husband's blood, even if found compatible by the accepted methods of testing, should never be used in a transfusion for his wife. This prohibition also affects his blood relatives, including his children. In this particular case, however, the nature of the incompatibility would not have been discovered if the husband's blood had not been tested. This suggests that the husband's blood and, if possible, also the child's blood, should be used in testing for antibodies in the blood of a woman giving birth to a child with hemolytic disease of the newborn.

One cannot fail being impressed by the rapidly increasing number of newly detected blood factors and it is reasonable to hope that the time will eventually come when every person will have his own individual blood formula.

APPLICATION OF BLOOD GROUPS TO EXCLUSION OF PARENTAGE

Inheritance of Blood Groups and Types

The blood factors are transmitted from parents to children according to Mendelian laws of inheritance. This is the basis for the application of blood groups and types to exclusion of paternity. Knowledge of a few basic rules of inheritance is necessary for the understanding of this application.

1. Properties transmitted by parents to children by inheritance are divided into two classes: (a) Dominant properties which are apparent, readily detectable and never hidden, and (b) recessive properties which may be hidden and not detectable by available tests in the individual

who received them by inheritance. These recessive properties which are not detectable in a person may be transmitted by him and become apparent in a descendant.

2. A and B are dominant properties. When present they must be recognizable or apparent and cannot be hidden.

3. Property O is recessive: it may be hidden.

4. A child inherits only one blood factor from each parent.

5. The individual who inherited the same blood factor from both parents is called homozygous; he is called heterozygous if he inherited a different blood factor from each parent.

6. *The first law of inheritance of blood group factors: The factors A or B cannot appear in a child unless they are present in one or both parents.*

The child cannot inherit them from anybody else but from a parent. To be transmitted by the parent they must be present in the blood of one or both parents, and if present in the parents' blood, being dominant properties, they must be apparent and recognizable by the available methods.

7. Property O can appear in a child though it is not detectable in the blood of the parents and, therefore, seemingly absent in them.

Actually this is also due to inheritance of property O, from one or both parents and not a new appearance in the child. In such a case both parents may be A, or B, or one may be A and the other B. In any case both of them are heterozygous: they have a recessive O which each of them transmitted to the child. The child received the property O from each parent in whom it was hidden.

8. *The second law of inheritance of blood group factors: A person of group AB cannot have a child of group O.*

A child gets one of its properties from each parent and both make up its blood group. A person of group AB received the A from one of his parents and the B from the other. Having both A and B he has no place for the recessive O in his blood. Therefore, he does not have the O and cannot transmit to his child the factor O but must transmit to the child either factor A or B. Let us suppose that the child inherited from the other parent property O. Having inherited from the first parent property A or B, the property O received from the other parent will obviously be hidden. For that reason a parent of group AB, regardless whether a father or mother, cannot have a child of group O.

9. *The third law of inheritance of blood group factors: A parent of group O cannot have a child of group AB.*

A child of group AB inherits the A factor from one parent and the B from the other. Therefore, it cannot have a parent of group O.

While the validity of the first law of inheritance of factors A, B, and O is unquestioned, an exclusion on the basis of the second and third laws is accepted by some authorities with reservation. The reason for that is two reported instances of a child O born to a mother of group AB, subgroup A_2B. Exclusion on the basis of the second or third laws is considered strong evidence that paternity is very unlikely. However, according to some writers, if the putative father belongs to subgroup A_1B and the child to group O the exclusion has the same validity as exclusions on the basis of the first law. Five-sixths of exclusions possible by means of blood groups are made on the basis of the first law, and only one-sixth on the basis of the second and third laws.

TABLE 6.14

Inheritance of ABO Factors

I. Law of Inheritance

Parent A can transmit only A or O (O = absence of A)
Parent B can transmit only B or O (O = absence of B)

hence

A and B cannot be present in the blood of a child unless present in the blood of one or both parents.

II. Law of Inheritance

Parent AB must transmit to child A or B

hence

Person AB cannot be parent of child of group O (because O denotes absence of A and B)

III. Law of Inheritance

Parent O can transmit to child only O, but not A or B

hence

Person O cannot be parent of child of group AB.

Inheritance of MN Factors

Parent M must transmit factor M to his child.
Parent N must transmit factor N to his child.

hence

IV. Law of Inheritance

M and N cannot appear in a child unless present in one or both parents.

V. Law of Inheritance

Parent M cannot have child N.

VI. Law of Inheritance

Parent N cannot have child M.

Exclusion of Paternity on Basis of Blood Factors M and N

The factors M and N are inherited as Mendelian dominants similarly as factors A and B. The differences between the inheritance of the blood groups A, B, AB, and O and the blood types M, N, MN are due to two facts:

(1) Every person must have one of the type factors M (type M) or N (type N) or both (type MN), while over 40 per cent of the population lack both A and B.

(2) Both factors M and N are dominant, while in the blood group system ABO the O is recessive.

Formulation of rules of inheritance may simplify the understanding of their application to exclusion of paternity. Some of the rules are identical with those formulated previously for the factors A, B and O.

(1) M and N properties are dominant. When present they must be recognizable or apparent and never hidden.

(2) A child inherits always one property from each parent, never more and never less.

(3) A parent of type M must transmit the factor M to his child; a

TABLE 6.15

Parents	Children Possible	Children Not Possible
O × O	O	A, B, AB
O × A	O, A	B, AB
O × B	O, B	A, AB
A × A	O, A	B, AB
B × B	O, B	A, AB
A × B	O, A, B, AB	
O × AB	A, B	O, AB
A × AB	A, B, AB	O
B × AB	A, B, AB	O
AB × AB	A, B, AB	O

TABLE 6.16

Parents	Children Possible	Children Not Possible
M × M	M	N, MN
N × N	N	M, MN
M × N	MN	M, N
M × MN	M, MN	N
N × MN	N, MN	M
MN × MN	M, N, MN	

parent of type N must likewise transmit the factor N to his child; a parent of type MN can transmit to his child only one of the two factors, either M or N.

From the three rules the following laws of inheritance of the blood factors M and N have been deduced.

First law: Factors M or N cannot appear in the blood of a child unless present in the blood of one or both parents.

This law is self-evident and identical with the first law of inheritance of blood group factors A and B.

Second law: A type M parent cannot give rise to a type N child; a type N parent cannot give rise to a type M child.

This law is easily understood if one recalls rule (3) that a parent who has only one factor M or N must transmit it to his children. Both laws have absolute validity. No proved exception has been reported. The recent discovery of the new factor S which is related to the MN system was discussed previously (see p. 301).

What are the chances of a man falsely accused of being the father of a child to prove his innocence? When the factors A, B, and O are used alone the chance is about 1 in 6 or about 16 per cent, but when both systems A, B, O and M, N are applied the chances are doubled, about 1 in 3 or about 33 per cent.

The above figures are based on large series. The figures vary depending on the blood group and type of the person. The rarer his blood group and type, the greater his chances; for instance, they are only 7.7 per cent for a man of the blood formula AMN and 64.3 per cent for a man with the rarest combination ABN.

In actual practice the exclusions are fewer because some of the accused men are the fathers. It must be emphasized that if a man accused of being the father of a child cannot be excluded as a possible father by means of grouping tests, the result does not mean that he is the father, because any man of the same blood group and type combination can be the father. For that reason only exclusion of paternity should be used in the courts as evidence, failure of exclusion should not be introduced as evidence, because it may be interpreted, without any justification, against the interests of the defendant.

The validity of the above laws of inheritance of the group factors A, B, O and of the type factors M, N has been tested in large series. Exclusion of paternity based on them has been accepted in the courts of many countries in Europe and in some states in this country. In 15 states (New York, New Jersey, Wisconsin, Maryland, Ohio,

Maine, South Dakota, North Carolina, Pennsylvania, Michigan, Indiana, Massachusetts, New Hampshire, Rhode Island, Oregon) laws have been passed authorizing the use of blood grouping tests in cases of disputed paternity.

Investigations are being conducted to further increase the chances of exclusion of paternity. One of the advances is based on the inheritance of subgroup A_1, A_2, A_1B and A_2B. The first studies conducted by Landsteiner and Levine were continued by Thomsen and associates who proposed a theory of inheritance of subgroups based upon the concept that A_1 and A_2 are dominant over O, and A_1 is dominant over A_2. This means that A_2 may be hidden and not detectable in a person just as O may be, but it can be so hidden only in a person of subgroup A_1.

Exclusion of paternity based on the heredity of subgroups does not as yet have the same validity as the exclusions discussed previously. Further studies are needed. The application of Thomsen's hypothesis adds only 3 per cent of possible exclusions to those possible by means of the other blood factors.

The most recent development in this field has to do with the discovery of the various Rh types.

The data published thus far permit formulation of the following laws of inheritance of the Rh types:

(1) Blood properties Rh_0 (D), rh′ (C), rh″ (E), hr′ (c), and hr″ (e) cannot appear in the blood of an individual unless present in the blood of one or both of his parents.

(2) Parents who lack the rh′ (C) factor cannot have children without the hr′ (c) factor; nor can parents without the hr′ (c) factor have children without the rh′ (C) factor.

(3) Parents who lack the rh″ (E) factor cannot have children without the hr″ (e) factor; nor can parents without the hr″ (e) factor have children without the rh″ (E) factor.

The fact that the ability to secrete is inherited as a Mendelian dominant permits its application to exclusion of paternity. The principle is that a secretor child of a non-secretor mother cannot be the child of a non-secretor father.

Technic of Tests for Exclusion of Paternity

Thorough familiarity with the technic of blood grouping and typing is a prerequisite for the performance of these tests. Their interpretation involves a knowledge of deviations from the normal and of genetic

aspects of the subject. The variations of sensitivity of the agglutinogens of the red cells, especially of properties A and N and of the agglutinating properties of the testing serums, especially of the anti-M and anti-N testing fluids, make it necessary to use not less than three grouping and typing serums of each kind and of red cell controls of known groups, which must include cells of groups A_1 and A_2, B and O, and of types M, N, and MN.

That blood grouping is the only scientific and reliable means of excluding paternity and of doing justice to men falsely accused is the consensus of all familiar with this subject in its immunologic, genetic, and medico-legal implications. It is their hope and wish that all the States of the Union will in time follow the example of the ten States that have already introduced laws governing the use of grouping tests in courts. It is obvious that performance of these tests, interpretation of the results, and testimony in courts should be entrusted only to qualified experts.

There are occasions when blood grouping tests can be used for exclusion of maternity, especially in conflicts arising from alleged interchange of infants in maternity hospitals.

Detection of Blood Groups and Types in Blood Stains and Stains Made by Secretions

The detection of blood groups in blood stains is made possible by the circumstance that factors A, B, and M are highly resistant to drying, heating and to the action of other physical and chemical influences. Iso-agglutinins are also resistant but to a lesser degree.

The examination presupposes that the stain was identified chemically as blood and as human blood by means of the precipitin test (see Section on Identification of Human Blood by Precipitins). The latter should include testing with a specific precipitating serum for human hemoglobin as recommended by Hektoen and Boor.

The study of the blood groups includes the demonstration of the agglutinins, if they are detectable, and of the agglutinogens. Factor N is less resistant than M and, therefore, less likely to be identified in older stains.

The blood group factors A and B are present in large amounts in secretions like saliva and sperm of many persons and can be frequently identified. The identification of the blood factors A and B in blood stains and in stains made by secretions is based on their ability to absorb iso-agglutinins from properly selected human serums.

The decrease of the titer of the agglutinins in the serum after addition of material scraped off the stain is an indication of the presence in the stain of the corresponding agglutinogen. The need for careful controls does not need to be emphasized. For the detection of property M in blood stains (it is not present in stains caused by secretions) a rabbit anti-M immune serum is absorbed.

It is not intended to discuss here details of technic. This work can be done reliably only by persons with experience in the special technics required for these procedures.

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CHAPTER 7

BLOOD CHEMISTRY

By Clarence Cohn and Alex Kaplan

GENERAL CONSIDERATIONS

The chemical measurement of various blood constituents is but one portion of a comprehensive examination designed to disclose the nature of a disease process. Blood analyses furnish data which, when integrated with the facts obtained from a complete history and physical examination of the patient and with other laboratory studies, aid the physician in making a diagnosis, in estimating prognosis and in following the results of therapeutic measures. Thus, chemical tests become part of a well-designed examination and should not be requested indiscriminately nor substituted for a thorough work-up.

In view of the multiplicity of tests available and the rising cost of laboratory examinations, it is essential to *plan* the work carefully and to utilize screening procedures whenever possible. This will avoid the expense of numerous and unnecessary tests which could become a financial burden to the patient. After results of the screening procedures have been examined critically in the light of the other data, additional or more specific tests can be outlined in an intelligent and economical fashion.

Many factors enter into a thorough understanding and interpretation of laboratory results. It is necessary to have some knowledge of the techniques employed by a particular laboratory, their degree of precision, the range of normal values with respect to age and sex and the alterations usually encountered in disease states. Only with such knowledge of the reliability and limitations of a chemical method and of the variations in concentration of blood constituents in normal and pathological conditions can laboratory results be fully evaluated. A list of the commonly accepted normal values appears in Table 7.1.

In order to avoid changes in composition, it is necessary to observe certain precautions in the drawing and handling of blood specimens prior to analysis. This imposes a specific obligation upon the intern, resident or attending physician to see that the laboratory obtains blood samples drawn under optimal conditions. The most accurate chemical analysis of a blood specimen that is handled improperly has no clinical value whatsoever.

Blood for analysis, with the exception of a postprandial blood sugar (see Glucose Section), should be taken at a time when the patient has been without food for 12 hours, preferably in the morning before breakfast. Such a procedure minimizes the influence of food ingestion, exercise and posture upon the concentrations of various blood constituents. Since stasis quickly causes changes in concentration, venous blood should be drawn without delay once a tourniquet has been applied. Hemolysis interferes with many tests and can be avoided if the following precautions are taken: (1) using a dry syringe, (2) removing the needle from the syringe and expressing the blood gently against the side of the collecting tube and, (3) avoiding the introduction of air bubbles. True values for pH, carbon dioxide content, chloride and sodium can be obtained only by drawing the blood without stasis (no tourniquet) and by preventing the loss of CO_2. This latter is done by placing mineral oil in the syringe, drawing the sample with a minimum of suction and transferring it to a tube while immersing the needle tip under a layer of oil. Analyses should be started as soon as possible after the withdrawal of blood in order to prevent the loss of glucose and other components by enzymatic, bacterial or oxidative processes. When blood is allowed to stand in a warm room as much as 20 mg. per 100 ml. of glucose may be lost per hour by glycolytic action of cellular enzymes. Some of the other changes that may occur are the breakdown of nitrogenous constituents, the alteration of electrolyte composition of serum as a result of an increase in red cell permeability, and an increase of inorganic phosphate at the expense of erythrocyte organic phosphate. When analyses cannot be started immediately, serum or plasma should be separated from the cells within an hour after collection and refrigerated until used. If a sample is deproteinized and the protein-free filtrate stored at refrigeration temperature, it will keep without deterioration for 24 hours or more.

It is preferable to use serum rather than whole blood or plasma for practically all measurements of chemical constituents except those of ascorbic acid, amino acid nitrogen, fibrinogen and blood oxygen content. In many instances, the three media are not equally desirable nor interchangeable. The authors' preference for serum may be summarized as follows: All blood constituents are divided unequally between red cells and plasma. Although the difference in distribution is not large for glucose and urea which are readily diffusible in body water, the concentration of all other substances differ markedly in red cells and plasma. Variations in the number of red cells (anemia or polycythemia) profoundly influence the results. Hence, the use of whole blood provides

a less sensitive medium than plasma or serum and many of the changes taking place are obscured by the dilution effect of the red cells. In some tests, red cells contain nonspecific substances that react to give falsely elevated values. Whole blood cannot be used for the performance of clearance studies (urea, creatinine, etc.) since it is plasma that is ultrafiltered at the glomerulus. Plasma is not desirable because anticoagulants such as sodium citrate or potassium oxalate cause shifts of water from cells to plasma which may dilute the plasma components as much as ten or fifteen per cent. Although a mixture of potassium and ammonium oxalates is effective and minimizes water changes (Heller and Paul), its use is limited because it cannot be employed in the determination of potassium, urea, non-protein nitrogen or calcium. Heparin is the most satisfactory anticoagulant but its expense has discouraged its use in routine laboratories. The uniformity of procedure and economy of blood provided by taking all analytical samples from the same fluid medium (serum) are distinct advantages.

When results are interpreted, it should be realized that for each constituent a state of dynamic equilibrium exists between tissue cells and the fluids that surround them. All substances are constantly entering and leaving the blood stream; their concentration at any given moment represents a balance between the summation of production (absorption from the gastro-intestinal tract, synthesis in the body or release from storage) and the summation of utilization (excretion, storage, breakdown or conversion to other forms). Blood, therefore, acts as a transport medium for compounds whose concentrations represent the net effect of the dual processes of production and utilization. Thus, it is possible for the concentration of a substance to rise either by increased ingestion or production or by decreased utilization or elimination. The converse is true for a decrease in concentration of a blood component.

It can be seen that unless blood for chemical analysis is drawn under standard conditions (i.e., fasting, with the patient at bed rest and on a constant diet) some physiologic process may cause an alteration in the dynamic state of equilibrium and hence in concentration of various substances in the blood. Since these conditions are rarely fulfilled one need not be surprised at some variation in concentrations from day to day, even in normal individuals.

It is the purpose of this chapter to indicate those clinical conditions where certain chemical determinations are of value in establishing a diagnosis, estimating prognosis and in following treatment. Where possible, results are interpreted in terms of dynamic, physiologic processes.

TABLE 7.1

Concentration of Serum Constituents of Fasting Normal Persons

Constituent*	Range
Amino acid nitrogen†	3.5–6.0 mg./100 ml.
Amylase	80–150 units (Somogyi)
Bilirubin	0.1–1.0 mg./100 ml.
Calcium	9.0–11.5 mg./100 ml.
CO_2 capacity	25–31 mM./L.
Chloride	100–106 m.e./L.
Cholesterol	
Total	120–260 mg./100 ml.
Esterified	68–76% of total
Creatinine	0.6–1.3 mg./100 ml.
Fatty acids, total	250–500 mg./100 ml.
Glucose‡	70–105 mg./100 ml.
Glucose, postprandial	less than 140 mg./100 ml.
Lipase	0.8–1.5 units (Alper) 0.2–1.5 units (Cherry-Crandall)
Lipids, total	470–750 mg./100 ml.
Phosphatase, acid	0–2.5 units (King-Armstrong) 0–1.5 phenol units
Phosphatase, alkaline	
Adults	1.5–4.0 units (Bodansky) 1.0–3.5 phenol units
Children	5–14 units (Bodansky) 4–12 phenol units
Phospholipids	150–350 mg./100 ml.
Phosphorus, inorganic	
Adults	3.0–4.5 mg./100 ml.
Children	4.5–6.0 mg./100 ml.
pH	7.35–7.45
Potassium	4.0–5.0 m.e./L.
Proteins	
Total	6.0–7.8 g./100 ml.
Albumin	3.2–4.1 g./100 ml.
Alpha Globulin	0.7–1.5 g./100 ml.
Beta Globulin	0.7–1.3 g./100 ml.
Gamma Globulin	0.7–1.3 g./100 ml.
Sodium	138–145 m.e./L.
Triglycerides	0–250 mg./100 ml.
Urea nitrogen§	11–17 g./100 ml.
Uric acid	3.5–6.0 mg./100 ml.

* The volume of serum necessary for analysis will vary, depending upon the specific method employed by each laboratory.

† The concentration listed is for plasma.

‡ Whole blood glucose is 60–90 mg./100 ml.

§ Whole blood urea nitrogen is 10–16 mg./100 ml.

For more details, the reader is referred to texts by Peters and Van Slyke, Cantarow and Trumper, or Everett.

A number of methods of varying degrees of complexity, accuracy and time consumption are available for the determination of each blood constituent. Of these, only one or two precise methods that are particularly well suited for multiple determinations in a large hospital laboratory are referred to in this chapter. These have been selected by the authors on the basis of personal experience in hospital and research laboratories, and although others may be equally good, the ones mentioned are quite satisfactory and convenient to carry out.

Details of analytical procedures may be obtained from original articles or from standard laboratory manuals or texts. The American Association of Clinical Chemists now publishes, "Standard Methods of Clinical Chemistry." Hawk, Oser and Summerson present a good account of the theory and applications of colorimetric measurements.

NITROGENOUS CONSTITUENTS

The compounds in serum that contain nitrogen in their structure may be classified into two main types, protein and non-proteins. The latter group, consisting of a heterogenous mixture of substances of relatively small molecular size that are not precipitated by certain protein precipitants (e.g., tungstic acid or trichloroacetic acid), is composed of the following constituents: urea, creatinine, creatine, uric acid, amino acids, ammonia and an unidentified fraction designated as "undetermined nitrogen." In this section, a discussion of the nitrogen-containing serum constituents is limited to those that have been found to yield useful clinical information when their concentrations have been altered. For an extended discussion of the metabolism of various non-protein nitrogen constituents, see the "Symposium on Azotemia" by MacFate et al.

Creatinine

Metabolism. Creatine (methyl-guanidoacetic acid), the precursor of creatinine, arises in the body primarily by synthesis although a small amount is ingested preformed in meat. In the kidney, the amidine portion of arginine combines with glycine to form guanido-acetic acid which is then methylated in the liver to form creatine. The labile methyl group is supplied by methionine, choline or other methyl donors. These steps are shown schematically in Figure 7.1. The creatine thus formed is transported mainly to muscle cells where it is phosphorylated to creatine phosphate (phosphocreatine), a compound of great importance in the contraction mechanism since it serves as a source of energy.

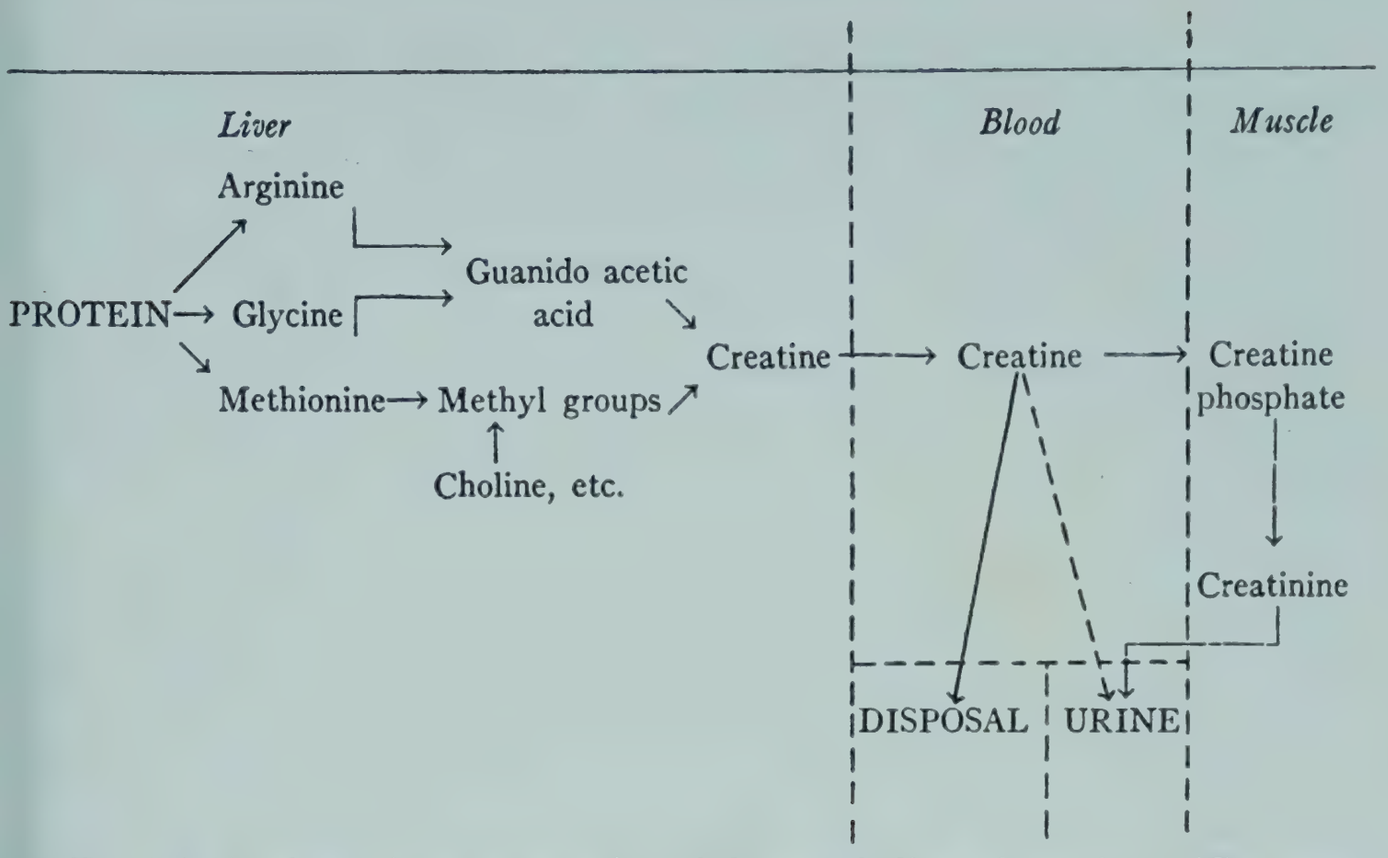


FIG. 7.1. A schematic representation of the metabolism of creatine. (Courtesy of Peters and Van Slyke, "Quantitative Clinical Chemistry, Interpretations", Vol. 1, 2nd Ed., The Williams & Wilkins Co., 1946).

Creatine appears in the plasma of adult males in small concentrations but is present at slightly higher levels in the plasma of women and children. Women usually excrete slightly more creatine than men while it appears normally in appreciable amounts in the urine of infants and children.

Creatine usually does not appear in the urine unless the plasma level exceeds 0.6 mg. per 100 ml. The serum concentration of creatine is not measured, however, since no useful clinical information is derived from it.

Approximately 2 per cent of the muscle creatine phosphate is converted to creatinine daily, the total amount depending upon the muscle mass. A small amount of preformed creatinine is ingested as a constituent of meat but this exogenous source has no perceptible effect upon serum levels. Creatinine is readily diffusible and is rather evenly distributed in body water. It is excreted primarily by the kidney which clears the plasma of creatinine at approximately the glomerular filtration rate; only small amounts are eliminated in the feces.

The concentration of creatinine in serum is not affected by diet, protein catabolism, age, sex or exercise. Elevated concentrations occur only when renal function is impaired by factors intrinsic or extrinsic to the kidney.

The same type of diagnostic and prognostic information concerning

renal function is obtained from the determination of either urea N or creatinine. The latter measurement is preferred by the authors, however, for the following reasons: (1) The determination of creatinine provides more accurate information concerning the state of kidney function because its serum level depends upon its rate of elimination since its endogenous production is relatively constant. Although there is evidence that there is some depression in the rate of formation of creatinine when serum levels rise to a concentration above 6 mg. per 100 ml., there are even more variations in the rate of protein catabolism and urea formation.

Whereas the serum concentration of urea is greatly influenced both by dietary protein and the rate of protein breakdown in the body, these factors do not affect creatinine concentration. These extrarenal variables detract from the value of a serum urea concentration alone as a measurement of kidney function. (2) Serum is cleared of creatinine by the kidney at the glomerular filtration rate, providing the basis for a good clearance test. Urea is cleared at approximately 60 per cent of the glomerular filtration rate because of its back-diffusion in the tubules.

Normal Concentration. Normal values for serum creatinine vary from 0.6 to 1.3 mg. per 100 ml. In most routine methods for the determination of creatinine, the colored compound produced by the reaction between creatinine and alkaline picrate is measured. This Jaffe reaction is nonspecific, however, and forms colored compounds with certain unidentified substances that are confined primarily to red cells. Using enzymatic methods for specifically measuring creatinine, Miller and Dubos compared such values with those obtained by the Jaffe reaction in normal and nephritic individuals. They found that *true* creatinine of serum comprised approximately 90 per cent of the "total" while in erythrocytes, the corresponding value was only 40 per cent. This emphasizes the need for using serum.

Increased Concentration. A concentration of serum creatinine over 1.5 mg. per 100 ml. definitely indicates impairment of urine formation or excretion. Minor changes in concentration are of significance because of the small fluctuations usually encountered. Clinical conditions that are characterized by an azotemia* caused by excessive protein

* In this chapter, *Azotemia* is defined as any pathologic condition, renal or extrarenal in origin, that is characterized by an elevation in the serum concentration of urea and/or creatinine. *Uremia* is defined as a clinical syndrome that may appear in severe, intrinsic renal disease in which the serum concentrations of urea and creatinine are very high as a result of impaired excretion; in addition, the renal lesion may cause the abnormal retention or elimination of other serum constituents.

breakdown show little increase in serum creatinine. The serum concentrations of urea N and creatinine tend to rise in parallel fashion in kidney disease when the protein intake and catabolism are constant.

Since creatinine is eliminated almost entirely in the urine, its concentration in serum rises when there is either insufficient formation or excretion of urine, irrespective of the cause. Normal urine formation and elimination depends upon (a) an adequate circulatory system that provides a sufficiently high rate of blood flow above a critical pressure to the functioning units of the kidney, the nephrons, (b) the presence of a sufficient number of intact and functioning nephrons, and (c) an unobstructed collecting and excretory system. Thus, a variety of conditions that depress the function of nephrons, alter their structure, or cause changes in extrarenal organs may produce a state of insufficient excretion of products normally discharged by the kidney; this is reflected by a rise in serum creatinine (and urea) concentration. It should be emphasized that an increase in serum levels of creatinine (or urea) does not necessarily indicate a disease process in the kidneys; factors extrinsic to the kidney may be responsible for the elevation.

For the purpose of classification, the various pathologic processes in which an elevated serum creatinine may be found have been divided, as follows, into those of renal and extrarenal origin:

A. *Intrinsic Kidney Factors:* Renal excretory activity represents a summation of the action of individual nephrons. Renal insufficiency may be produced by the partial loss of function of a large number of nephrons or by the complete loss of function of a smaller number of nephrons; the degree of the insufficiency and the magnitude of the azotemia which it produces therefore depend upon the number of nephrons concerned and the extent of the involvement.

The nephron may be damaged by a lesion in any part—the glomerulus, the tubule, or in the blood supply to these parts. Pathological processes in the interstitial tissue may interfere with the function of nephrons by compression of the blood supply or by altering glomerular or tubular function. Rarely, however, is the damage over a period of time confined to one area of a nephron since a pathologic process usually progresses and involves other sites. Most kidney diseases have concomitant tubular and glomerular dysfunction even though one type of lesion may predominate. Nephrosclerosis, for example, is a primary renal vascular disorder that causes glomerular obliteration and eventual tubular degeneration. Lesions in the glomeruli also predominate

in the acute stage of glomerulonephritis; with chronicity, however, tubular function likewise becomes impaired. Diseases in which the original lesion may have been primarily confined to the interstitial tissue (chronic pyelonephritis, amyloid disease) gradually progress until finally there is tubular and glomerular involvement. As the glomerular filtration rate decreases, the concentration of serum creatinine rises. In acute renal failure* following traumatic and toxic injuries (crushing injuries, burns, transfusion reactions, surgical shock, poisoning with sulfonamides, mercuric chloride, carbon tetrachloride, etc.), severe tubular damage is found. Trauma produces an ischemia which results in random tubular disruption; poisons cause a necrosis of the epithelial cells of the proximal tubules and in addition, a shock-like state that may produce the ischemic changes.

Abnormalities in tubular function can result from demonstrable lesions, as described above, or from some metabolic defect in the tubule cells. Thus the impaired ability of the tubules to reabsorb sodium chloride may lead to dehydration which, as described below, may cause an azotemia through the depression of the glomerular filtration rate.

B. *Extrarenal Factors:* The elevated serum creatinine and urea levels found in conditions where extrarenal factors interfere with either urine formation or elimination are usually referred to as "pre-renal" or "post-renal" azotemia.

1. Pre-renal Azotemia: A reduction in glomerular filtration rate and renal blood flow is the basis for the rise in serum creatinine and the other abnormalities found in this type of retention. The physiologic causes for the reduced renal blood flow and glomerular filtration may be listed as follows:
   a. Congestive heart failure.
   b. Salt and water depletion caused by:
      (1) Vomiting, diarrhea, gastro-intestinal fistulas.
      (2) Excessive sweating with deficient salt intake.
      (3) Diuresis associated with uncontrolled diabetes mellitus or diabetes insipidus.

* Acute renal failure has frequently been referred to as "lower nephron nephrosis." Oliver, MacDowell and Tracy have demonstrated that the condition is neither a nephrosis nor is the lesion confined to the distal convolution. These workers propose that the incorrect term of "lower nephron nephrosis" be replaced by either "acute renal failure" or "ischemuric episode."

(4) Addison's disease, primary, or secondary to anterior pituitary insufficiency; in addition a decrease in intraglomerular blood pressure further depresses urine formation.

c. "Shock." In addition to a decreased blood flow, the lowered intraglomerular pressure also interferes with glomerular filtration. Prolonged shock may produce the ischemic tubular changes described under acute renal failure.

2. Post-renal Azotemia: Obstructions of the urinary tract that prevent the elimination of urine produce an azotemia. The degree of azotemia depends upon the completeness and duration of the obstruction. With a mechanical obstruction in the urinary tract, urine continues to be formed by the kidney until a hydrostatic pressure is finally built up in the tubules that is sufficiently great to inhibit or prevent glomerular filtration. Examples of this are: (a) prostatic hypertrophy, (b) neoplasms compressing both ureters, (c) bilateral calculi, or (d) congenital abnormalities (torsion or kinking of ureters, urethral valves, aberrant renal arteries compressing the renal pelvis).

Decreased Concentration. Low serum creatinine values have no clinical significance.

Indication for Test. The determination of urea N or creatinine should be carried out whenever diminished kidney function is suspected. It should be borne in mind, however, that other tests such as the urea or the creatinine clearance, etc. (see Renal Function, Chapter 9) are more sensitive and more quantitative indicators of impaired renal function. When elevation of serum creatinine does occur, it is important for prognosis to determine the trend of this change by repeated analyses at regular intervals. It is of little value to estimate prognosis or to attempt to establish a specific diagnosis upon the basis of a single creatinine determination; the trend of its serum level and all of the clinical data must be examined critically and viewed in its entirety.

Method. A yellow-red color is produced in a deproteinized filtrate of serum by the action of alkaline picrate upon creatinine. The method of Bonsnes and Taussky is recommended.

Volume of Serum. 1 ml.

Urea Nitrogen

Metabolism. Urea is the principal end-product formed fron the catabolism of protein. It is extremely diffusible and consequently is dis-

tributed evenly in the water of all tissues. With the possible exception of its mild diuretic action, however, urea normally has no useful function in the body and is excreted almost entirely by the kidneys. The urea in plasma is filtered by the kidney glomerulus but under normal conditions approximately 40 per cent of this filtered portion back diffuses into the blood. The clinical importance of urea arises from the fact that an elevated concentration in the blood stream may be associated with impaired renal function.

In 1932, Krebs and Henseleit demonstrated the general pathway by which ammonia is converted to urea in mammals. These workers described the ornithine cycle in which ornithine combines with ammonia and CO_2 to form citrulline which in turn reacts with more ammonia to produce the amino acid, arginine. The liver enzyme, arginase, hydrolyzes arginine to urea and ornithine, leaving the latter free to participate again in the cycle.

Recent work (Ratner) has expanded the original concept of Krebs and Henseleit by revealing the sources of ammonia, CO_2 and energy by which ornithine is converted successively to citrulline and then to arginine. As shown in Figure 7.2, glutamic acid is necessary for the formation of citrulline while the necessary energy is supplied by the high energy phosphate bonds of ATP (adenosinetriphosphate). The ammonia is derived from the deamination of amino acids. The conversion of citrulline to arginine is more complicated, however, and requires the participation of the tricarboxylic (citric acid) cycle. Aspartic acid combines with citrulline to form an intermediate compound which splits into arginine and malic acid, leaving the amino group of aspartic acid incorporated in the arginine molecule. The tricarboxylic cycle supplies the aspartic acid as well as the energy for the reactions through the generation of high energy phosphate bonds. Thus, urea is formed within the framework of an ornithine cycle by a complicated process involving the formation of many intermediate compounds.

Arginine is produced in many tissues but since the enzyme, arginase, is found almost exclusively in the hepatic cells of mammals, the liver is the only detectable source of body urea. The ability of hepatic cells to form urea is one of the last functions to fail when extensive hepatocellular damage occurs.

Since the end-product of analysis is usually measured as ammonia nitrogen, it is customary to express the concentration of urea in terms of its nitrogen (urea N)[3]. Urea concentration in the blood stream is determined by the balance between the rate of protein breakdown and the

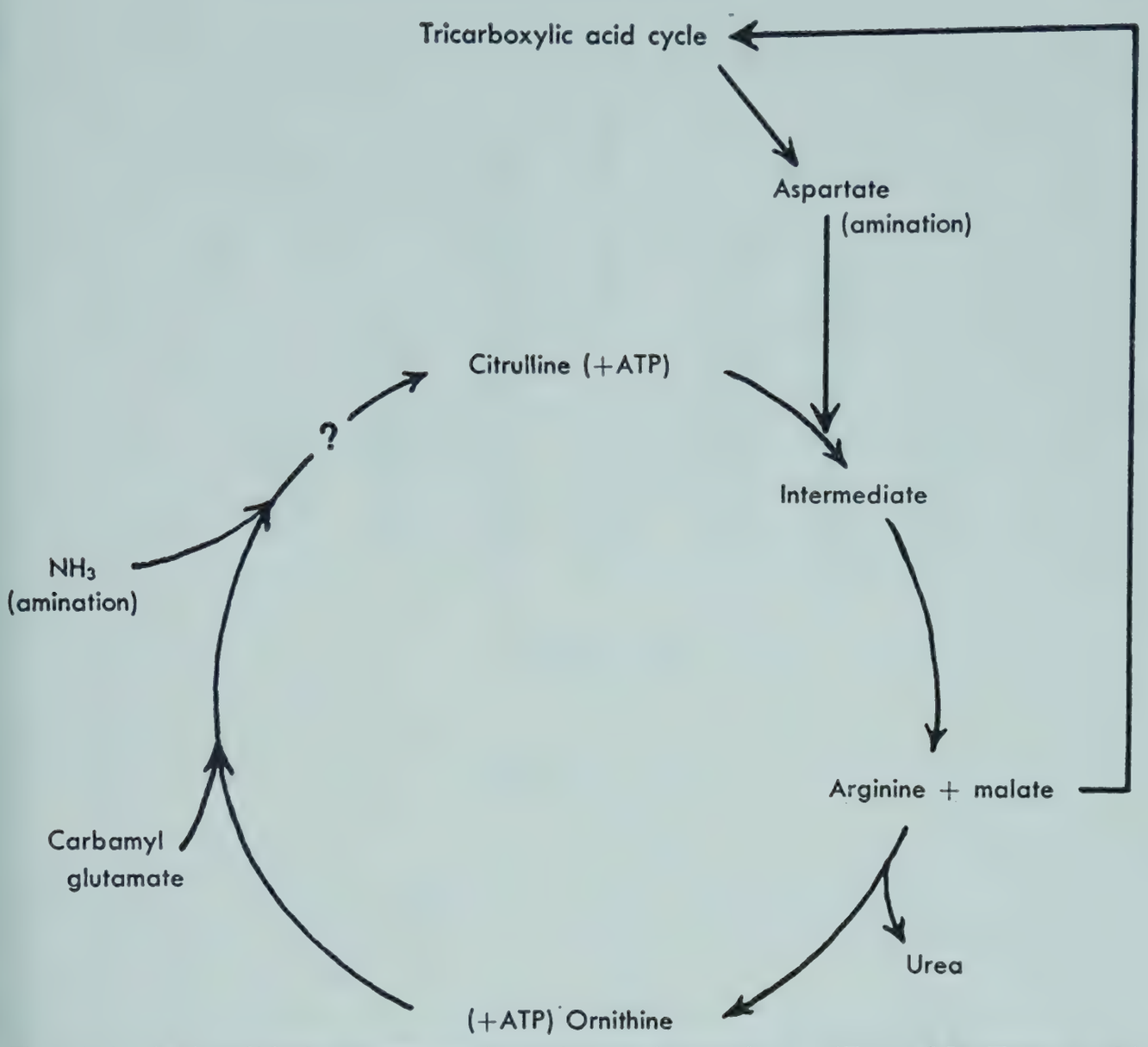


FIG. 7.2. A simplified representation of urea formation. (From MacFate *et al.*)

rate of elimination by the kidney. The capacity of the body for utilizing exogenous protein for synthesis or storage is limited when the diet is adequate in both calories and protein. In these circumstances, the extra protein ingested is catabolized, as evidenced by an increase in serum urea and in the urinary excretion of urea and ammonia.

Addis and his coworkers have emphasized that the amount of protein in the diet has a profound influence on the serum level of urea in normal persons as well as those with kidney disease. Figure 7.3 illustrates the direct relation between the amount of protein in the diet and the serum level of urea N. In a similar manner, the excessive breakdown of tissue proteins raises the serum urea level.

Normal Concentration. The normal range for serum urea N* extends from 9 to 19 mg. per 100 ml., depending to a large extent upon the protein intake, while the commonly accepted one for whole blood is

* To convert values of urea N to urea, multiply by 2.14.

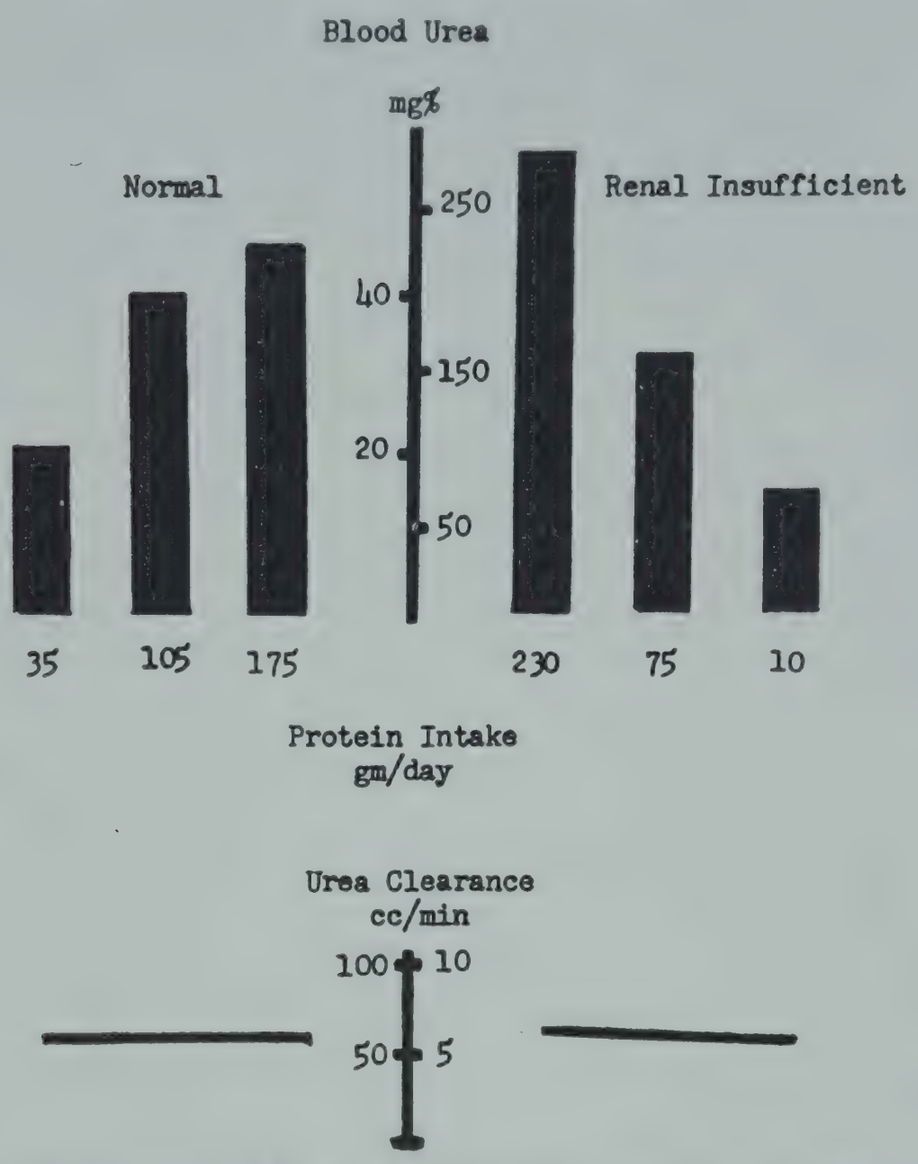


FIG. 7.3. The influence of dietary protein intake on blood urea concentrations. (MacFate *et al.*)

9 to 17 mg. per 100 ml. Age and sex have little influence upon urea N concentration; in the absence of pathologic processes, the chief determinant is the amount of protein ingested and catabolized.

Increased Concentration. Elevated urea N occurs in a number of pathological conditions and the cause may be ascribed to increased production in the course of accelerated protein catabolism, to decreased elimination as a result of impaired kidney function or to a combination of these two processes. It is the common teaching that substantial renal damage must occur before the retention of urea with raised serum levels (azotemia) is found. Goldring, however, maintains that on a constant nitrogen intake, blood urea rises with the first reduction in urea clearance. He considers the former concept to be erroneous and that many conclusions are invalid because (a) too high an "upper limit of normal" blood urea levels are accepted, with the result that small but significant increases are missed and (b) variations in nitrogen and water balance obscure the true relationship between urea concentration

and urea clearance. With a constant protein intake, the degree of azotemia depends upon the extent and type of kidney damage. The elevated urea N provides no information concerning the nature or location of the process involved, i.e., whether caused by failure of glomerular filtration, abnormally high tubular reabsorption or blockage of the urinary collecting system.

The principal conditions in which an increased concentration of serum urea N may be found are the same as those listed for creatinine. In addition, a mild elevation may occur in the absence of renal involvement if excessive amounts of protein are broken down. This may happen in cases of stress, myocardial infarct, a severely bleeding peptic ulcer, etc.

Decreased Concentration. A lowered serum urea N concentration caused by decreased production characterizes only a few conditions. These are listed below:

a. *Protein malnutrition.* This may follow diminished intake or impaired absorption of protein.
b. *Pregnancy.* During the last few months, values as low as 5 mg. of urea N per 100 ml. may be encountered.
c. *Premortal stage of acute yellow atrophy.* In the late stages of this disease, the serum urea concentration falls while that of amino acids rises because of the failure of the ornithine cycle and deamination and transamination reactions.

Indication for Test: The serum level of urea provides the same type of information concerning kidney function as that of creatinine but the latter is preferred for reasons given above.

Method. The method of Conway is recommended as an accurate, simple titrimetric method. Serum is placed in the outer chamber of a Conway cell where the enzyme, urease, converts urea into ammonia and CO_2. After alkalinization, free ammonia diffuses into the boric acid solution in the center well. The ammonia is titrated directly with standard acid and the amount of urea originally present can be calculated. MacFate et al. discuss various other procedures for the determination of urea N.

Volume of Serum. 0.2 ml.

Non-Protein Nitrogen

As mentioned earlier in this section, non-protein nitrogen (NPN) is a term applied to the nitrogen derived from a heterogeneous mixture of compounds. The composition of this mixture is variable and contains some compounds that have not been identified.

For clinical purposes, the NPN determination should be eliminated in

favor of urea N or creatinine. Although it yields the same type of information as that obtained from urea N or creatinine levels, it is an inferior substitute since the latter tests measure specific substances and can be performed more readily and accurately. NPN, moreover, is derived from a group of substances that may vary independently of each other and in different directions, although the main factors that determine its concentration are often the same as those that effect urea N.

Uric Acid

Metabolism (Bishop and Talbott). Uric acid is a purine compound that circulates in human red cells and plasma, with its concentration in the latter being approximately twice as high as in the cells. In man and ape, uric acid is the principal end product of purine metabolism and is excreted in the urine, but other mammals further oxidize the compound to allantoin. In humans, uric acid arises from ingested nucleoproteins (purine derivatives), from the degradation of nucleoproteins in cellular material, and by synthesis from simple, chemical precursors. Figure 7.4 illustrates the main steps in the formation of uric acid from the breakdown of nucleoprotein. As shown in Figure 7.5, the sources of nitrogen for the synthesis of uric acid are glycine and ammonia from the metabolic pool while the carbon atoms are provided by glycine, formate and CO_2. Studies with isotopic uric acid indicate that approximately 0.7 to 0.85 gm. are produced daily by persons receiving a low purine diet. This amount is larger than the daily urinary excretion of uric acid on this diet and may have to be revised downward since there is no evidence

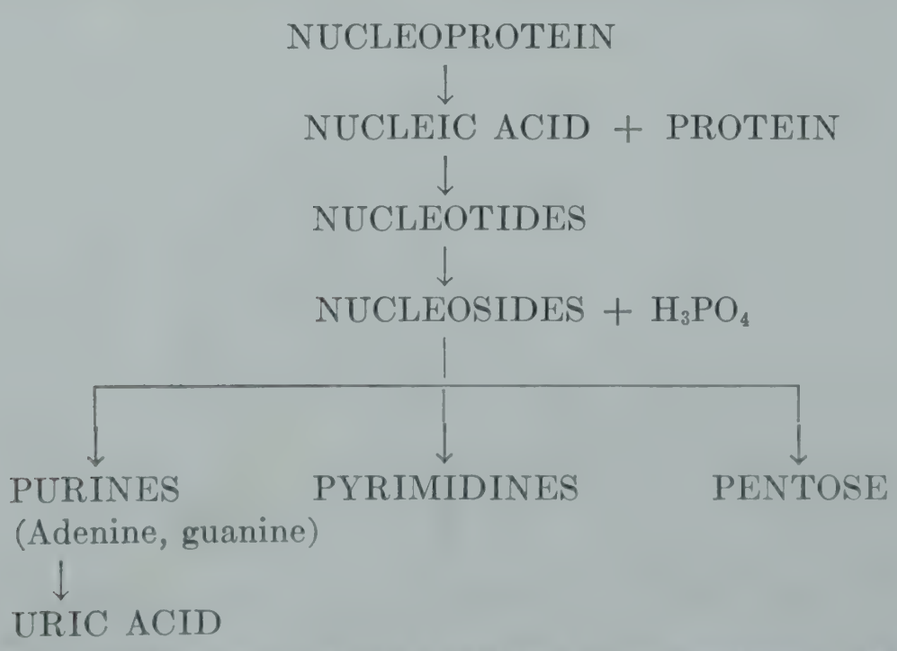


FIG. 7.4. Stepwise formation of uric acid from nucleoproteins. (Modified from MacFate *et al.*)

for further breakdown of uric acid in the body except by intestinal bacteria.

The sites of uric acid synthesis in man are not known although the liver is essential for the final conversion of other purines to uric acid. The bone marrow, gastro-intestinal tract and liver are organs that have a high turnover of nucleoprotein and are probable sites of purine synthesis.

Although ingested uric acid may be broken down by bacteria in the intestinal tract, human tissue cells do not appear to destroy it and the major portion is eliminated in the urine. Some uric acid may be removed from the blood by the liver and eliminated through the intestinal tract by way of the bile. The studies with isotopic uric acid failed to include an investigation of feces or bile, which leaves the question still unsettled. Present concepts concerning the metabolism of uric acid are contained in a Combined Staff Clinic Report (Am. J. Med.).

As with other serum constituents, the concentration of uric acid depends upon the net balance achieved between the rate of synthesis of purines or breakdown of nucleoproteins on the one hand and the rate of elimination of uric acid on the other. In the presence of a normally functioning kidney, the ingestion of nucleoproteins has little influence upon the serum concentration of uric acid unless very large amounts are taken. Variation in serum levels may be explained by changes in production or elimination rates or by a combination of these processes.

Normal Concentration. The normal concentration of uric acid varies from 3.5 to 6.0 mg. per 100 ml. for normal adult males; in females the normal value is generally about 15 to 20 per cent lower. Normal values

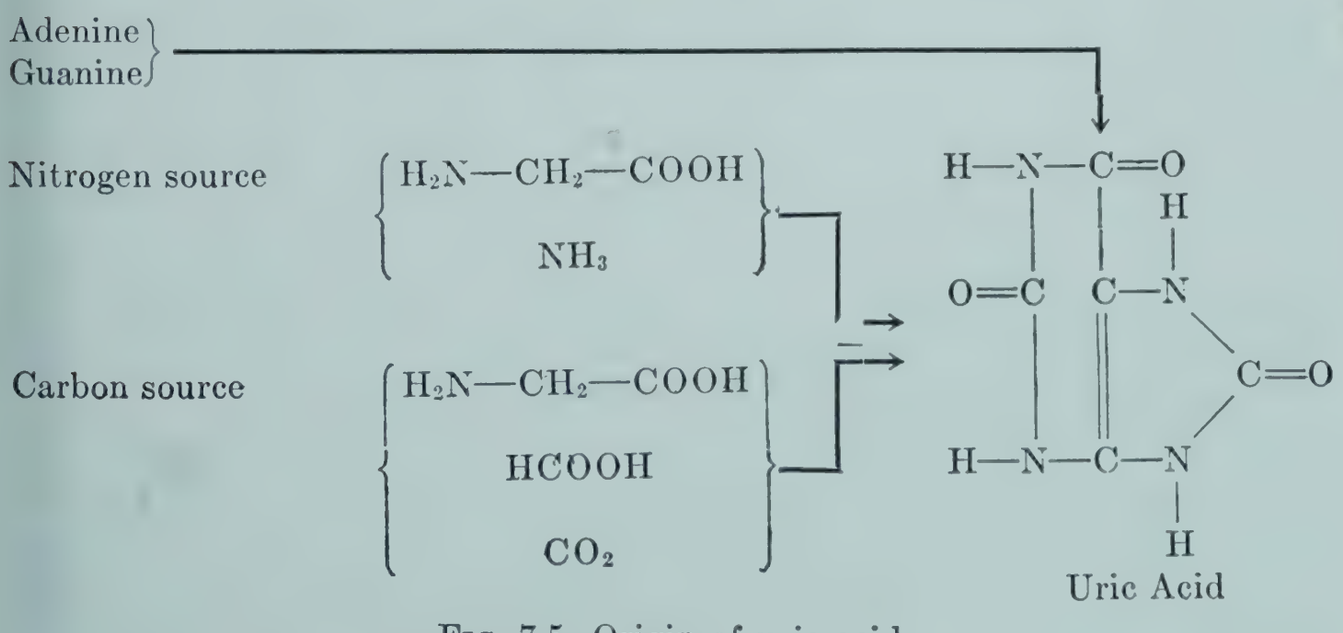


FIG. 7.5. Origin of uric acid

for children are lower even than those of normal women and this difference persists until the age of puberty; children show no sex difference in serum uric acid concentration. For men, women and children, the average values per 100 ml. of serum are 5.1, 4.1 and 3.3 respectively. Although chemical methods employed for the measurement of uric acid are non-specific and include a small percentage (about 10 per cent) of chromogen that cannot be acted upon by uricase, the enzyme that specifically splits uric acid, the ratio in serum of chromogen to true uric acid is constant in health and disease. For this reason, Wolfson et al. feel that the chromogen is as much a part of nucleoprotein metabolism as true urate and should be included in the analysis.

Increased Concentration. The most common disorder of purine metabolism is *gout*, a disease characterized by an increased serum level of uric acid; it occurs much more frequently in males than in females. According to Bauer, hyperuricemia is almost invariably present, with only 2 per cent of gouty patients having serum concentrations below 6.0 mg. per 100 ml. Serial determinations are sometimes necessary to establish a diagnosis. Another feature of chronic gout is the deposition of urate in tissues; this process may occur not only in the area of joints but also in renal parenchyma, where the ensuing inflammatory reaction may produce in time a secondary renal insufficiency. In addition, some cases of gout are associated with arteriolosclerosis. The impaired kidney function becomes evident when a creatinine clearance test is performed. It is not known whether the hyperuricemia of gout is caused by deficient elimination or increased production of uric acid. The depressed uric acid clearance of gouty patients may be raised by the administration of uricosuric drugs (cincophen, colchicine, salicylates, Benemid,* etc.) and this has been interpreted as signifying impaired elimination; however these drugs also raise the urate clearance of normals. It is possible that the increased serum uric acid level and decreased urate clearance in gout may be associated with an increased fraction of circulating urate bound to protein or existing as a polymer instead of the free form. On the other hand, studies with isotopic uric acid demonstrate that some gouty patients have a greatly increased pool of miscible urate and imply that the cause of the disorder may lie with increased production from endogenous sources. Apparently there are two types of gouty patients, those with an abnormally high basal uric acid excretion and those with a normal excretion. Only the former group has an accelerated synthesis of uric acid from labelled glycine (Benedict et al.).

* p-(di-n-propylsulfamyl)-benzoic acid.

The role of the adrenal cortex in purine metabolism and gout is not well defined. ACTH or adrenal cortical extracts lower serum uric acid by increasing renal excretion.

Asymptomatic hyperuricemia is a frequent finding in the relatives of patients with gout, apparently on the basis of a genetically inherited characteristic. Hyperuricemia, arising from an excessive destruction of nuclear material, is seen frequently in *leukemia*, *polycythemia*, *resolving pneumonia*, and *toxemias of pregnancy*. In the last named condition, serial serum uric acid analyses are sometimes of value in estimating prognosis; a steady and progressive rise is usually regarded as an indication for interrupting the pregnancy.

High serum uric acid levels as a result of decreased elimination are common in severe kidney dysfunction of various types. There is no uniform correlation, however, between the serum concentration and the severity of kidney damage. Consequently, uric acid determinations should not be used for estimating the extent of renal impairment. The retention of urea and creatinine appear earlier, are more marked and are of greater value in diagnosis and prognosis.

Decreased Concentration. A hypouricemia is of no clinical significance. It will be found after the administration of ACTH and uricosuric drugs (which include aspirin).

Indication for Test. The level of uric acid in serum should be evaluated whenever gout is suspected. During toxemias of the latter half of pregnancy, repeated determinations aid in following therapy and in estimating prognosis.

Method. Uric acid reacts with arsenophosphotungstic acid in alkaline solution to give a blue color which is intensified by cyanide. A modification of the method of Buchanan, Block and Christman is suitable for serum.

Volume of Serum. 1 ml.

Amino Acids

Metabolism. Amino acids are the fundamental structural units which are linked together in large numbers to form proteins. There are some 21 to 23 amino acids of biological significance; variations in their number or type of combination produce the multitude of individual proteins. Naturally-occurring amino acids have an amino group on the alpha carbon atom and, in the body, prove to be compounds of extreme reactivity, participating in a host of reactions.

Ingested protein is the body source of amino acids. In the gastrointestinal tract, proteins are hydrolyzed to amino acids or peptides which

are absorbed and transported to the liver where many of the metabolic reactions involving these acids occur. In the liver, the amino acids may participate in the synthesis of protein, urea, uric acid, creatine, etc., may become deaminated or may be utilized as a source of energy. A portion of them, however, passes through the liver unchanged and is carried by the blood to extrahepatic tissues. Tissue cells, in turn, deaminate or transaminate amino acids, utilize them for the synthesis of particular proteins, exchange them for other amino acids in proteins and even contribute to the amino acid pool by the hydrolysis of protein (Schoenheimer). Only the liver, however, is capable of forming urea from the ammonia residues (see section on urea N). The metabolism of amino acids is represented schematically in Figure 7.6. It can be seen that the pathways of anabolism and catabolism are manifold and that fragments of amino acids can turn up in a large number of compounds. In general, the nitrogen portion of the molecule finally leaves the body as one of the non-protein nitrogen constituents of urine, principally as urea. Although small amounts of amino acids are excreted in the urine, the major portion is metabolized within the body.

Normal Concentration. The normal concentration of total amino acid nitrogen (AAN) in plasma is low, varying from 3.5 to 6.0 mg. per 100 ml.; greater concentrations are found in erythrocytes. Since this represents the concentration of amino nitrogen contributed by some 22 different acids, it is obvious that the composition of the group can vary

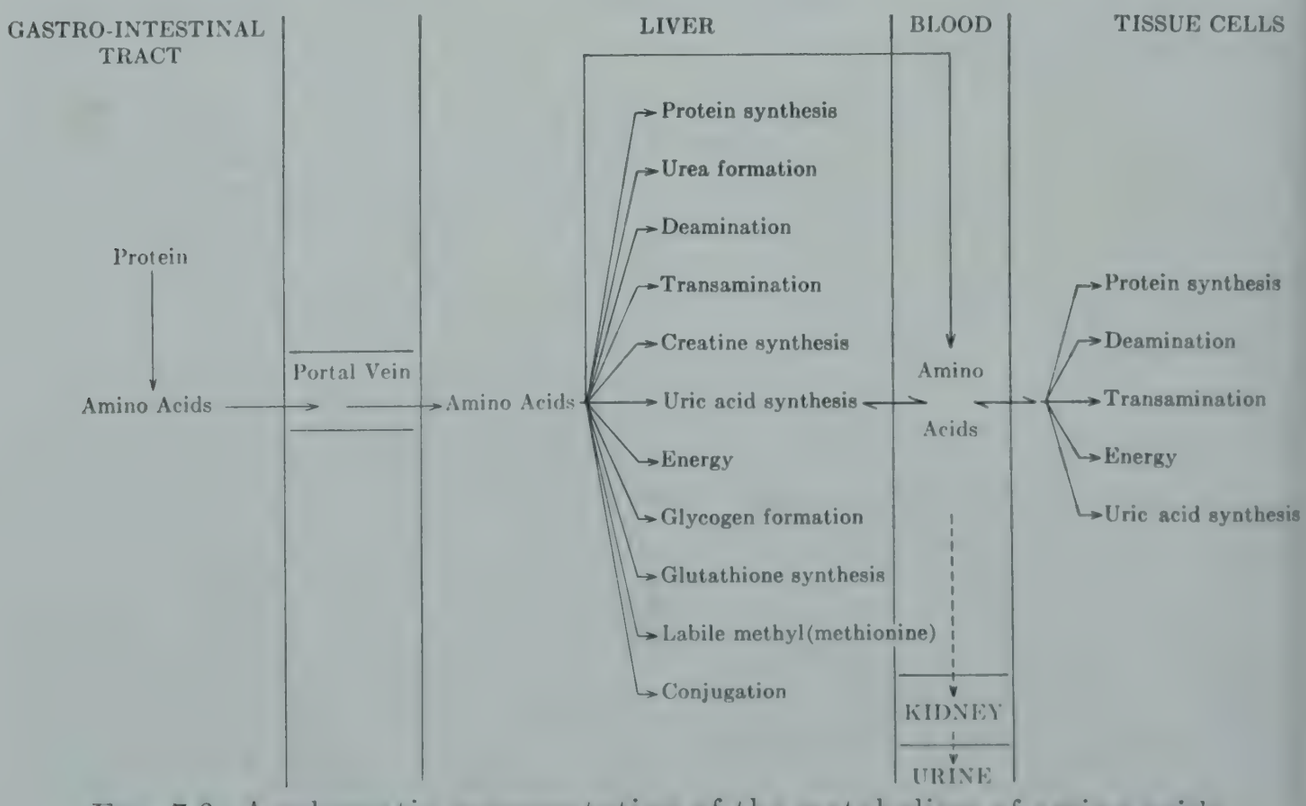


FIG. 7.6. A schematic representation of the metabolism of amino acids

greatly without a detectable change in concentration. Although it is possible to measure the concentration of many of the individual amino acids by microbiological procedures, such a complicated, tedious process is not suited for a routine chemical laboratory; in addition, there are insufficient data available for clinical interpretation. At present, only two amino acids in plasma—glutamine and glycine—can be determined readily by chemical methods and these together account for about 25 to 35 per cent of the total AAN. As yet, their measurement is of value only in special studies and has no place in clinical diagnosis. Only the determination of total AAN is of clinical significance at present and its use is limited. Peters and Van Slyke (1946) provide an excellent discussion of the metabolism of individual amino acids.

Increased Concentration. AAN of plasma is increased in several conditions but in only one, *acute yellow atrophy*, does it aid in establishing a differential diagnosis. The high concentration found in terminal stages of this disorder may be ascribed to overwhelming hepatic destruction, leading to failure of deamination as a step leading to urea synthesis. In hepatitis and cirrhosis, there is usually sufficient liver function to handle deamination and other reactions involving amino acids. The infrequent increases reported in *eclampsia* may result from both accelerated protein breakdown and disordered hepatic function.

The administration of ACTH or C_{11} oxysteroids raises the plasma level of AAN, which is probably a reflection of the increased mobilization of protein from the periphery. A protein meal is followed by a small and transitory rise in AAN.

Decreased Concentration. Lowered levels of AAN are seen in the crisis of the *nephrotic syndrome*, during the crisis of *pneumonia*, and following *severe injury*. This fall has never been explained in a satisfactory manner. The administration of insulin, epinephrine and growth hormone lower plasma AAN by increasing peripheral removal.

Indication for Test. If overwhelming hepatic parenchymal destruction is suspected, serial serum AAN concentrations may be of value in confirming the diagnosis. During the crisis of the nephrotic syndrome, a lowered level of AAN may indicate the need for plasma or parenteral amino acids.

Method. In this determination, the use of plasma is preferred to serum since amino acids are released in the clotting process, producing higher values.

The most accurate method for the determination of plasma amino acids is the gasometric analysis of Van Slyke, Dillon, MacFadyen and

Hamilton but this is usually too time-consuming for use by a routine laboratory. The colorimetric method of Frame, Russell and Wilhemi, as modified by Russell, is more convenient and is sufficiently accurate for clinical purposes.

Principle (Frame et al.). Amino acids in a deproteinized filtrate of plasma produce a colored compound when heated with beta-naphthoquinone-4-sulfonic acid in alkaline solution. This is measured colorimetrically.

Volume of Plasma. 1 ml.

Ammonia Nitrogen

Interest in the peripheral blood ammonia levels has been revived in the past several years because of reported increases in the concentration of ammonia in cases of hepatic coma. The value of this test in individuals with severe hepatic damage is open to question, however. There is only a poor correlation between blood NH_3—N levels and mental state of any given individual with severe liver disease; coma may even precede the elevation in the concentration of this constituent in blood. Furthermore, no prognostic significance can be attributed to the concentration of NH_3—N. The very wide range of "normal" values (0 to 200 micrograms per 100 ml. of blood) that has been reported for NH_3—N indicates that the methods employed by the various investigators lack specificity. For these reasons, we see no need for performing this determination at the present time.

Serum Proteins

Metabolism (Gutman). One of the chief functions of the plasma proteins, particularly of the albumin fraction, is the maintenance of plasma oncotic pressure, one of the factors regulating the distribution of water between blood and tissues. As information concerning specific plasma proteins increases, it is evident that plasma proteins are more than inert osmotic regulators of blood volume. Hormones, antibodies, enzymes, and the proteins responsible for blood clotting are examples of specific plasma proteins having known functions. As newer methods of separation and purification of proteins are utilized, the functions of the individual plasma proteins are becoming clearer.

The sites of formation of only a few of the plasma proteins are known. Albumin, prothrombin and fibrinogen are probably formed only in the liver. On the other hand, the globulins originate to a far greater extent in extrahepatic tissue, as shown by the formation of protein hormones

in endocrine glands and the probable formation of gamma globulin in plasma cells or lymphoid tissue. There is considerable evidence to indicate that the circulating proteins are in a state of dynamic equilibrium with tissue proteins although the mechanisms for their interchange are not known. This is particularly true of albumin which seems to serve as a precursor of tissue and bone protein. During periods of protein starvation, cellular proteins may be sacrificed in order to maintain the circulating proteins. It has been demonstrated that, when protein is lost from the body, 30 gm. of cellular protein disappear for every gram of plasma protein. Replacement therapy for malnourished individuals must take this relationship into account even though there is no known charteristic alteration in plasma proteins which will indicate overall body protein stores.

In normal individuals approximately 0.2 to 0.3 gm. of plasma proteins are formed daily per kilo of body weight. This is equivalent to 15 to 20 gm. per day of new circulating proteins for a 70/kg. adult; since protein concentrations and blood volumes are rather constant, it follows that the same amount of protein is degraded daily. The average half-life of serum proteins is two weeks, but varies with the individual protein. Thus, prothrombin may have a half-life of only several hours. Nothing is known about sites of protein degradation, the factors which regulate rates of formation and utilization, or the conditions which control plasma protein concentration.

It is possible to fractionate the serum proteins by chemical techniques (salt-precipitation, alcohol precipitation, isoelectric precipitation, etc.), by physical methods (ultracentrifugation, chromatography, free or zone electrophoresis, etc.) and by immuno-chemical procedures. All methods have yielded fractions to which various names have been given. The terminology used to designate fractions originally separated by electrophoresis are the ones most commonly used in modern medical literature. The principle protein groups have been designated as albumin and as alpha, beta and gamma globulins. As better techniques of separation are perfected, the globulin fractions are being divided into subgroups, e.g., alpha one and alpha two, etc. The separation of proteins into these aggregates should not be misconstrued to mean there are only four circulating proteins. Actually some 75 to 100 plasma proteins are known at the present time and are demonstrable when individual components are separated from the large fractions.

Total Protein. Normal Concentration. In normal individuals the total serum protein concentration varies from 6.0 to 7.8 gm. per 100 ml.

It is much lower in the newborn infant, ranging from 4.5 to 5.5 gm. pe 100 ml. but it reaches adult levels in about 4 to 6 months (Smith) Since fibrinogen is converted to insoluble fibrin during the clotting proc ess, the concentration of total protein and total globulin is about 0.2 gm. per 100 ml. higher in plasma than in serum. Compared to indi viduals at bed rest, ambulatory patients may have a serum protein con centration that is 0.5 to 0.8 gm. per 100 ml. higher. This is a result o the pooling of fluid in the lower extremities when an individual assume the erect position. Estimation of the total amount of circulating pro tein has been employed only infrequently as yet, but yields information which is obscured by performing only the determination of concentration. Thus, some individuals with cirrhosis may have normal amounts of protein in circulation as indicated by an increase in plasma volume in spite of an apparent hypoproteinemia.

Indication for Test. Total protein concentration represents the sum total of numerous different proteins, many of which vary independently of each other and in different directions. The determination of total protein only supplies limited information except in conditions of *shock* and *dehydration*, where the concentration increases, or in *hemorrhage*, where the level tends to decrease. In these circumstances, total pro tein levels are of value as a guide for parenteral administration of fluids during an emergency. It is also useful to know the total serum protein level when measuring calcium concentration since the non-diffusible calcium in serum varies directly with the total serum protein. Aside from these conditions, more information can be gained by estimating the concentrations of serum albumin and total globulin. If abnormalities are revealed in these fractions, a complete protein fractionation should be carried out. As with other components of serum, an alteration in the concentration of the proteins will occur from an imbalance between the rates of production and utilization.

Albumin. Normal Concentration. "True" serum albumin (see meth ods), that portion of the serum proteins that corresponds to the albumin fraction obtained by electrophoretic separation, normally comprises 52 to 55 per cent of the total serum proteins or 3.2 to 4.1 gm. per 100 ml. of serum. Values for serum albumin obtained by the older Howe method (precipitation of globulin with 21.5 per cent Na_2SO_4) are about 30 per cent too high because alpha globulins are not precipitated by that salt concentration and are included with the albumins. The use of 26.9 per cent Na_2SO_3 as a precipitating agent (Wolfson, Cohn et al.) or 26.8 per cent Na_2SO_4 (Majoor, Milne) avoids this error.

Increased Concentration. Because of its relatively low molecular weight and rather high concentration in the blood stream, the albumin fraction exerts through its high oncotic properties more influence on plasma volume than any other plasma protein. Hence, conditions of hyperalbuminemia are rarely seen except in the presence of acute dehydration and shock since tendencies toward increased albumin concentration are masked by increased plasma volume.

Decreased Concentration. Causes for decreased albumin concentrations are not always clear since homeostatic processes operate to minimize alterations in plasma concentration. Although a certain amount of overlapping occurs, the pathologic conditions characterized by hypoalbuminemia may be classified as follows:

1. *Deficient intake of protein:* Lowered serum albumin concentrations are seen in *malnutrition*, *starvation* and in cases with long-standing pathology of the gastro-intestinal tract where there has been interference with the digestion and absorption of proteins: *sprue*, *celiac disease*, *cystic fibrosis of the pancreas*, *intestinal fistula*, *carcinoma of the intestinal tract* and *ulcerative colitis*. A decrease in serum albumin level may also occur in conditions which impose a demand for extra protein if the dietary intake of protein is insufficient: *pregnancy*, *lactation* and *hyperthyroidism with deficient caloric intake*.
2. *Deficient synthesis of albumin:* In *chronic liver disease*, especially in *cirrhosis*, the hypoalbuminemia is usually ascribed to impaired synthesis. There is evidence, however, that increased utilization may also be a factor. Impaired formation of albumin also occurs in *chronic infections* and in *uncontrolled pernicious anemia*.
3. *Excessive protein breakdown:* The inadequate caloric intake which often accompanies *uncontrolled diabetes mellitus* or *hyperthyroidism* may result in excessive breakdown of protein for use as a source of energy. In such instances, plasma albumin as well as tissue proteins are catabolized. Acute insults to the body in the form of *fever*, *infections* or *trauma* lower the concentration of serum albumin. In these stress situations, hypoalbuminemia may be caused by the increased protein catabolism that follows the accelerated release of C_{11} oxysteroids by the adrenal cortex.
4. *Loss of albumin:* The usual explanation advanced for the extremely low albumin concentration found in the *nephrotic syndrome* is the large loss of albumin in the urine. The amounts lost, however, cannot completely account for the low values, and it appears that there must be some concomitant defect in albumin synthesis,

increase in utilization, or both. During the acute stage of *glomerulonephritis* the moderately decreased concentrations may be due to adrenal cortical factors (see above). In the chronic phase of this disease, impaired synthesis and deficient protein intake play a part. Severe loss of protein by extravasation, as seen in *severe hemorrhage*, *extensive thermal burns* and "weeping" skin conditions (*extensive exfoliative dermatitis*) produces a hypoalbuminemia. Since the albumin of exudates is derived from plasma, disease states associated with *extensive edema* or *ascites* may also produce a moderate lowering of serum albumin concentration.

Total Globulin. Normal and Pathologic Values. The normal level of total globulin in serum varies from 2.6 to 3.8 gm. per 100 ml. At birth, the concentration is about 1 gm. per 100 ml. but this rises steadily until it approaches the adult range in about 4 or 6 months (Smith). The concentration of globulins increases in a variety of conditions, most of which are associated with an infectious or malignant process. The rise probably represents increased production. Elevated globulin concentrations can be expected in *bacterial pneumonia*, *malaria*, *syphilis*, *subacute bacterial endocarditis*, *tuberculosis*, *leukemia*, *multiple myeloma*, *rheumatoid arthritis*, *sarcoidosis*, *rheumatic fever*, *lymphopathia venereum* and some tropical diseases (*schistosomiasis*, *kala-azar*, *leprosy*, *filariasis*). Since the serum albumin concentration is normal or decreased in most conditions of hyperglobulinemia, the determination of only total serum protein may fail to uncover some abnormalities. It should be pointed out that the absolute values of albumin and globulin have far more significance than the A/G ratio which sometimes can be misleading in diagnosis. Since albumin and globulins are independent variables, alterations in the A/G ratio do not indicate whether the concentration of albumin, globulins or both are abnormal. Thus, the finding of an A/G ratio of 0.1 in the nephrotic syndrome signifies something entirely different from the occurrence of the same ratio in multiple myeloma. This ratio of 0.1 can occur with an albumin level of 0.35 and a globulin of 3.5 gm. per 100 ml. in the former condition or with an albumin of 1.0 and a globulin of 10 gm. per 100 ml. in the latter.

Alpha Globulins. In a protein fractionation, the serum globulins are subdivided into the categories of alpha, beta and gamma globulins. In the proper circumstances, this test yields useful clinical information. The physiological significance of the major globulin fractions is slowly being revealed as more of the individual protein components are being identified.

In the alpha globulin fraction are the smaller lipoproteins, some enzymes and the thyroxin- and copper-carrying moieties. These proteins can be estimated only by special techniques; chemical or electrophoretic techniques determine the entire alpha globulin aggregate. The normal level of alpha globulin is 0.7 to 1.5 gm. per 100 ml. and its concentration usually varies inversely with albumin. Thus, when the concentration of serum albumin decreases in *acute febrile disease*, *cirrhosis*, *malnutrition* and the *nephrotic syndrome*, alpha globulins rise. This inverse relationship does not hold true for *multiple myeloma* where the alpha globulin level may be normal despite a low albumin concentration.

Beta Globulins. This fraction contains the larger lipoproteins, some antibodies, enzymes and the iron-binding protein, transferrin (see below).

Increased levels of beta globulins are usually associated with hyperlipemias and hypercholesterolemias. Such levels are observed in the *nephrotic syndrome*, *idiopathic lipemia*, *uncontrolled diabetes* and *obstructive jaundice*. Elevations occur during normal pregnancy, a condition associated with a moderate increase in serum lipids. Sometimes the abnormal serum proteins appearing in multiple myeloma are included with the beta fraction; these abnormal proteins are found most frequently in the gamma and beta fractions and occasionally in the alpha portion.

Gamma Globulins. The gamma globulin fraction contains most of the immune bodies and is present in normal serum at a concentration of 0.7 to 1.3 gm. per 100 ml. In the serum of the newborn, the gamma globulin concentration is at adult levels but it then decreases continually for several months. It drops to 50 per cent of the concentration present at birth and then rises slowly, taking at least a year to reach the adult range (Moore et al., 1949). Pathologically high values are found in infectious diseases (such as *lymphopathia venereum*, *kala-azar*, *tuberculosis*, *leprosy*, in *acute and chronic glomerulonephritis*, *amyloidosis*, *sarcoidosis*, "*collagen*" *diseases* (*periarteritis nodosa*, *lupus erythematosus*, *dermatomyositis*), *hepatitis* (except in early stages), and in *multiple myeloma*). Serial determinations of gamma globulins are of value in differentiating between *obstructive* and *hepatocellular* types of jaundice since this constituent does not rise in the former condition.

Decreased concentrations of gamma globulins occur in the *nephrotic syndrome* and may drop as low as 0.1 gm. per 100 ml. Low values are also seen following *widespread destruction of lymphoid tissue* by x-ray or by nitrogen mustard poisoning, in *pyridoxine deficiency* and *idiopathically* in some patients.

Indication for Fractionation. A complete fractionation is of aid in

establishing a differential diagnosis between nephrosis and acute glomerulonephritis since gamma globulins are usually increased in the latter condition but markedly decreased in the former. Such a fractionation is of value in helping to distinguish between biliary obstruction and hepatitis; albumin and alpha and gamma globulins are normal in obstruction whereas albumin tends to be low and alpha and gamma globulins high in the presence of a damaged liver parenchyma. Fractionation studies also serve as a guide to prognosis in liver disease; both high gamma globulin levels and low or falling albumin values indicate poor prognosis. Recovery from these conditions cannot be considered complete as long as the protein fractions remain at abnormal levels.

In Table 7.2 are listed changes in protein fractions that are commonly found in various pathologic states.

Special Proteins: *Fibrinogen.* Fibrinogen is a circulating globulin; its conversion to fibrin is the end product of the clotting process. Fibrinogen has a normal concentration of 0.19 to 0.33 gm. per 100 ml. Its measurement, however, adds little diagnostic information that cannot be secured more readily by other determinations except in cases of *fibrinogenopenia.*

TABLE 7.2

Changes in Total Serum Protein and Protein Fractions in Various Diseases

Disease	Total Protein	Albumin	Globulin			
			Total	Alpha	Beta	Gamma
Rheumatoid arthritis	→	↓	↑	↑	→	↑
Lupus erythematosus	→↑	↓	↑	→	→	↑
Nephritis						
Acute	→	↓	↑	↑	↑	↑
Nephrotic syndrome	↓	↓	→↓	↑	↑	↓
Hepatitis						
Acute	→↓	↓	↑	↑	↑	↑
Chronic (cirrhosis)	→↓	↓	↑	↑↓	↑	↑
Biliary obstruction	→	→	→↑	→	↑	→
Acute infection	→	↓	↑	↑	→	↑
Chronic infection	→↓	↓	↑	↑	↑	↑
Malnutrition	→↓	↓	↓	→	→	↓
Multiple myeloma*	↑	↓	↑	→↑	→↑	→↑

A decrease in concentration is denoted by ↓, an increase and by ↑ and a normal value by →.

* The abnormal proteins of this disease may appear in the alpha, beta or gamma fractions.

Transferrin: Iron in plasma is transported in combination with a specific beta globulin, transferrin. One molecule of this protein is able to bind two atoms of iron. Normally, the total iron-binding capacity of serum is only one-third saturated. The determination of serum iron and the total iron-binding capacity is indicated in individuals suspected of having *hemachromatosis* since in this disease there is an increase in the saturation of transferrin with iron.

Ceruloplasmin: Copper in plasma is transported in combination with an alpha globulin, ceruloplasmin. In *hepatolenticular degeneration* (*Wilson's Disease*), there is a deficiency of this protein, with consequent low serum copper values. There is an elevated excretion of this ion and amino acids in the urine.

Method. The protein fractionation method of Wolfson, Cohn, Calvary and Ichiba is recommended because of its simplicity, precision and speed. Total protein and a complete fractionation into albumin, alpha, beta and gamma globulins can be carried out with 1.0 ml. of serum, yielding results that are in good agreement with electrophoretic measurements.

Volume of Serum. 1 ml.

Paper electrophoresis (Tiselius; McDonald) is now being used rather extensively for separating and measuring the various serum protein fractions. As difficulties in staining characteristics of the various protein groups and quantitation of concentrations are overcome, the usefulness of this technique will be enhanced.

CARBOHYDRATES

Glucose

Carbohydrate is important in the body economy as the chief fuel for body processes. Since the stores of this material in the body are limited (approximately 300 grams for a 70-kg. individual), the total amount present will fulfill the energy requirements for only about half a day. It is obvious that unless there is regular ingestion of this foodstuff, fat and protein will be converted to carbohydrate to satisfy body needs.

In the gastro-intestinal tract, poly- and disaccharides are enzymatically hydrolyzed to monosaccharides and absorbed either by diffusion or by some active cellular process (phosphorylation?). The rate of absorption varies with each individual sugar and is affected by the type and amount of food in the intestine, the emptying time of the stomach, the physiological state of the intestinal mucosa, and endocrine activity. In general, the state of thyroid activity and the salt-regulating functions of

TABLE 7.3

*Effects of Endocrines on Tissues and Blood Sugar**

Tissue	Hormone							
	Insulin		Thyroid Hormone		Adrenal Cortical Steroids		Anterior Pituitary Hormones†	
	Lack	Excess	Lack	Excess	Lack	Excess	Lack	Excess
Gastrointestinal absorption	→	→	↓	↑	↓ or →‡	→	↓ or →	↑ or →
Liver output of glucose	↑	↓ then ↑ §	↓	↑	↓	↑	↓	↑
Muscle uptake of glucose	→¶	↑	↓	↑	↓	↑	↓	↑
Blood sugar	↑	↓	→ or ↓	→ or ↑	↓	↑	↓	↑

* The following designations are used: ↑ = Increased rate or value; → = normal rate or value; ↓ = decreased rate or value.

† The effects of anterior pituitary hypo- or hyperfunction will be mediated thru its thyrotropic and adrenocorticotropic hormones. In addition, growth hormone in excess has an anti-insulin effect.

‡ When the sodium chloride content of the extracellular fluids is normal, normal gastrointestinal absorption occurs.

§ Insulin initially inhibits glucose release by the liver. If this is followed by hypoglycemia and therefore epinephrine release, glycogenolysis results.

¶ At the characteristically elevated blood sugar of the diabetic, the removal of glucose from blood is normal.

adrenal cortical hormones influence the absorption rate of carbohydrate while insulin has no effect (see Table 7.3).

Metabolism. The hexose, glucose, is the only sugar found in the blood of a normal, fasted individual; it is stored in the form of its polymer, glycogen. Glycogen, which represents approximately 70 per cent of the total carbohydrate in the body, is present only as an intracellular component; free glucose, in contrast, is found only in extracellular fluids to any appreciable extent.

Glucose is phosphorylated after passing through cell membranes, and is then able to enter metabolic processes. By means of a number of enzyme-catalyzed reactions, glucose can be stored as glycogen, be oxidized to CO_2 and water with the release of energy or be converted into fat or amino acids after degradation to two-carbon fragments.

Blood glucose concentration is normally maintained within a relatively

narrow range. This constancy is the result of a number of homeostatic factors which are in a state of delicate balance and which are sensitive to slight alterations in blood glucose levels (Soskin and Levine). These factors include the hepatic and renal uptake and release of glucose, glucose removal by peripheral tissues, and the effect of numerous hormonal influences upon these processes. Intestinal absorption of carbohydrate has only a temporary effect upon blood glucose levels.

The liver and to a lesser extent the kidney are the only endogenous sources of blood sugar. The former is the most important organ involved in the homeostatic control of blood sugar; it can both remove and contribute glucose to the blood as well as synthesize it from a number of amino acids and fatty acid fragments. Under certain conditions, the kidney can release glucose into the circulation but the mechanism for this is not clear.

In general, the blood sugar level itself serves as a stimulus to the liver and is a factor in determining whether glycogenesis or glycogenolysis shall predominate. Hyperglycemia, in the presence of normal amounts of insulin, inhibits the hepatic output of glucose while accelerating its deposition, both processes aiding in restoring the blood sugar to normal. Hypoglycemia is usually associated with decreased hepatic gluconeogenesis and glucose output. The endocrine glands-anterior pituitary, thyroid, islet cells of the pancreas, and adrenal cortex-influence blood sugar levels mainly by the effects their hormones exert upon the liver. Glucagon, a hormone (?) of the alpha cells of the pancreas, raises blood glucose levels by a glycogenolytic effect on liver glycogen. Physiologically, its role in the homeostasis of blood glucose is unknown. Epinephrine usually is not a factor in regulating blood sugar levels, but in emergencies, its increased secretion may cause excessive hepatic glycogenolysis and hyperglycemia. The effects of the endocrines on hepatic blood sugar regulation are listed in Table 7.3.

All peripheral tissues utilize glucose for energy purposes. Of these only skeletal and cardiac muscle are able to store significant amounts as glycogen. Within certain blood sugar limits, there is a direct relationship between blood glucose concentration and the utilization of carbohydrate by extrahepatic tissues. This relationship between blood glucose level and peripheral utilization holds for both normal and diabetic individuals although the rate of utilization is lower in the latter group at a given glucose concentration. In contrast to this, when hypoglycemia is present, the rate of entry of glucose into muscle will vary with the cause of the decreased blood sugar. Insulin is the one hormone

which has been shown to increase the extrahepatic uptake of glucose. This hormone apparently facilitates the transfer of glucose through cell membranes prior to its phosphorylation. Table 7.3 lists the effects of the endocrines on the uptake of glucose by muscle.

Normal Values. The serum glucose concentration of normal humans in the postabsorptive state varies from 70 to 105 mg. per 100 ml. while the corresponding values for whole blood are 60 to 90 mg. per 100 ml. The level is not influenced by age or sex. Infants born of either normal or diabetic mothers, however, exhibit a transient hypoglycemia during the first 12 to 36 hours after parturition.

All blood sugar concentrations mentioned in this chapter are "true" glucose values.

Increased Concentration. Abnormal blood sugar concentrations result from some imbalance between hepatic output of glucose, peripheral uptake of the sugar, or disturbances in the endocrine regulatory influences upon these processes.

Hyperglycemia may result from (1) an increase in hepatic production and release of glucose, with normal removal rate by peripheral tissues; (2) a normal hepatic output of glucose with a subnormal rate of peripheral removal; or (3) a combination of these factors.

The syndrome called *diabetes mellitus* is characterized by persistent hyperglycemia and glycosuria. Mild diabetics, however, may show normal fasting blood glucose concentrations and may require postprandial blood sugar determinations or the performance of a glucose tolerance test in order to establish a diagnosis, especially since glycosuria may be absent. The primary abnormality in the syndrome is an overproduction of glucose by the liver. Within limits, at any given blood glucose concentration, the diabetic organism utilizes less carbohydrate than a normal control. At his characteristically elevated blood sugar level, however, the diabetic utilizes carbohydrate at a rate comparable to a normal at the latter's usual blood sugar concentration. Glycosuria occurs when the renal tubular reabsorptive capacity for glucose is exceeded. The diabetic syndrome can result from an absolute or relative insulin lack. This lack may be caused by lesions in the islets of Langerhans or by disorders of other endocrine glands. Increased activity of the adrenal cortex, anterior pituitary, and to a lesser degree, the thyroid, may result in hyperglycemia and the diabetic syndrome. Increased adrenal cortical activity, either primary or as a result of *pituitary basophilism* with increased ACTH production, will elevate the blood sugar since the increase in hepatic gluconeogenesis is greater than the increase

in peripheral uptake. *Pituitary eosinophilism*, with overproduction of growth hormone leading to *acromegaly*, produces the diabetic syndrome, although the mechanism is not known. Growth hormone may exert its primary influence on hepatic gluconeogenesis. In addition, this hormone has an anti-insulin effect and may also effect glucagon production. Excess thyroid hormone may raise blood glucose levels if the rate of peripheral utilization cannot keep pace with the augmented hepatic output. Hyperglycemia may be seen in individuals with a *pheochromocytoma*, if the blood is drawn at a time when excess epinephrine is discharged from the adrenal medullary tumor. In the diabetic syndrome, insulin restores hepatic gluconeogenesis to normal and facilitates peripheral utilization at normal blood sugar levels.

Elevated blood sugar levels are encountered in a number of other conditions. *Anoxia* may cause hyperglycemia since liver glycogen is unstable in the presence of a deficient oxygen supply. *General anesthesia* also mobilizes liver glycogen and raises the blood sugar because of an attendant adrenal medullary stimulation, anoxia or acidosis. Probably the combination of anoxia and epinephrine secretion are responsible for the hyperglycemia seen in the convulsive episodes of *tetany*, *epilepsy*, *eclampsia*, and *intracranial trauma*. Damage to the floor of the fourth ventricle may contribute to the hyperglycemia seen in the last named condition. With *chronic hepatic dysfunction*, increased blood sugar values may occur because of the relative insensitivity of hepatic cells to the various homeostatic mechanisms. Only rarely will increased blood sugar levels be seen secondary to gastro-intestinal dysfunction. Usually, *gastrectomy* with resultant rapid passage of foodstuffs into the intestine ("dumping syndrome"), may be found to be the cause for this hyperglycemia.

Decreased Concentration. Hypoglycemia may result from (1) a decrease in hepatic gluconeogenesis, when combined with normal peripheral utilization of glucose; (2) normal hepatic glucose output with increased peripheral uptake; or (3) some combination of these two processes. Hyperinsulinism, caused by *pancreatic islet cell hyperplasia*, *adenoma*, or *carcinoma*, produces hypoglycemia. The low blood sugars result from an inability of hepatic glucose output to keep up with the accelerated rate at which it is removed by the periphery. Insulin increases the peripheral uptake of glucose and favors glycogenesis by the liver but the counter-regulatory effects of hypoglycemia increase the hepatic glucose output. Hyperinsulinism may be demonstrated by the presence of a low, fasting blood sugar, by exercise after a 12 to 18 hour fast or by the

performance of an intravenous glucose tolerance test (see glucose tolerance section). The usually occurring hypoglycemic phase of the test is exaggerated under these conditions, especially if the test is carried out for 4 to 6 hours. With *hypofunction* of the *adrenal cortex*, *anterior pituitary*, or *thyroid gland*, there is an imbalance between peripheral removal and hepatic output. All these states of endocrine deficiency are characterized by a decrease in the rate of both hepatic formation of glucose and peripheral removal but the depression of the former predominates, with the appearance of lowered blood sugar levels. *Overwhelming hepatic destruction*, following phosphorus, benzol, chloroform, carbon tetrachloride, or arsphenamine poisoning, may result in hypoglycemia since the organ that is the main source of the blood sugar is destroyed. With a less severe degree of hepatic dysfunction, either hypo- or hyperglycemia may be seen. *Glycogen storage disease* (Von Gierke's) is characterized by a hypoglycemia, since the increased hepatic stores of glycogen are not available for blood sugar formation. Although some *psychoneuroses* are characterized by hypoglycemia, the functional abnormality in such conditions has not been clarified. *Methyl alcohol poisoning* in "smoke" drinkers is often associated with depressed blood glucose values and evidence of a pancreatitis.

Indication for Test. There is no need for estimating the level of serum glucose in the fasting state, except where hypoglycemia is suspected or where it is desired to ascertain the effect and duration of insulin action in diabetics under therapy. In estimating serum glucose levels, one is attempting to screen out individuals with disorders of carbohydrate metabolism leading to hypo- or hyperglycemia. A fasting serum glucose determination will aid in establishing the former situation, especially when combined with exercise. On the other hand, disease states associated with hyperglycemia can be more readily demonstrated when tested under the load of a meal. Fasting blood glucose levels *are not* the estimation of choice in screening individuals for the presence of diabetes (see postprandial blood glucose section).

In addition to persons suspected of hypoglycemia, it is also advisable to determine the blood sugar level in the following situations: (1) cases of unexplained coma in order to exclude uncontrolled diabetes; (2) patients exhibiting convulsions, especially during a convulsive episode since hypoglycemia may frequently be confused with tetany or epilepsy; (3) individuals with fatigue, to determine whether hypoglycemia is a factor.

Method. Serum proteins are precipitated and the glucose in the filtrate is oxidized with hot alkaline cupric or ferricyanide solutions.

The amount of reduced copper is estimated by iodometric titration or by colorimetric measurement of the blue color that is formed in molybdate solution. Ferrocyanide produced by reduction with glucose is usually measured colorimetrically after reaction with Fe^{+++} to form "Prussian Blue."

In addition to glucose, such substances as glutathione, ergothioneine, uric acid, etc. which are found in red cells and plasma, will also reduce copper solutions. For the preparation of protein-free filtrates for glucose analysis, $Zn(OH)_2$ or $CuWO_4$ should be used since the non-glucose reducing substances will be precipitated by these protein precipitants.

Tungstic acid, the precipitant employed in many routine laboratories, should be abandoned because it fails to precipitate these non-glucose reducing substances and blood sugar values obtained on this filtrate may be 20 to 70 mg. per 100 ml. too high.

The authors recommend the Nelson glucose method for its accuracy and for the ease with which large numbers of analyses can be carried out.

Protein precipitant: Acid-$ZnSO_4$ solution* + $Ba(OH)_2$. By placing an aliquot of serum in acid-$ZnSO_4$ solution, the sample can be stored in a refrigerator for several days if necessary without loss of glucose by glycolysis.

Glucose oxidation: With alkaline Cu^{++} solution at 100°C.

Color development: With arsenomolybdate reagent. The color produced is stable for a day or two.

Volume of Serum. 0.5 ml. or 0.1 ml. if desired.

Carbohydrate Tolerance Tests

The ability of an individual to regulate his blood sugar can best be studied by determining blood glucose concentrations after a meal or after oral or intravenous glucose. The blood glucose response to the

* Modification introduced in the authors' laboratory:

Acid-$ZnSO_4$ solution. Dissolve 4.0 gm. of $ZnSO_4 \cdot 7H_2O$ in water, add 15 ml. of N H_2SO_4 and make up to 1 liter volume.

0.35 N $Ba(OH)_2$. Dissolve 55 gm. of $Ba(OH)_2 \cdot 8H_2O$ and make up to 1 liter volume. Filter and store in bottle protected by means of a soda lime tube. Balance the $Ba(OH)_2$ solution against the $ZnSO_4$ by titrating 50 ml. of the latter with the former to a phenolphthalein end-point. If the reagents are properly balanced, this titration should require 5.88 ml. of $Ba(OH)_2$. Dilute the more concentrated of the 2 solutions until there is exact balance.

Protein precipitation. To 8.5 ml. of acid-$ZnSO_4$, add 0.5 ml. serum and mix. Add 1 ml. $Ba(OH)_2$, mix and centrifuge. The remainder of the procedure is carried out according to the direction of Nelson.

load of carbohydrate can be used to evaluate the over-all mechanism responsible for the homeostasis of the blood sugar. The postprandia blood glucose determination requires only one specimen 1 to 2 hour after a meal; only the degree of hyperglycemia is of importance. Wit the glucose tolerance test, the concentration of blood glucose is de termined at specified time intervals before and after the oral or intra venous administration of a given amount of glucose. With the ora test, both the degree and duration of the hyperglycemia are of clinica importance, while in the intravenous method, only the duration of the hyperglycemia is significant.

Physiology. The liver is the organ primarily involved in the regula tion of blood sugar and therefore is the one most concerned with the type of blood sugar response to a load of carbohydrate, whether administered as part of a meal or as glucose orally, or intravenously. When given orally, glucose produces an elevation in the blood sugar since it is ab sorbed at a rate which at first is greater than the capacity of the liver and peripheral tissues to remove it. As a result of adjustments, the blood sugar is restored to normal after a time, although the intestinal absorption may be continuing at a maximum rate. Although it is assumed that there is a "normal" absorption of glucose from the intestine, even in normal individuals this absorption rate is somewhat variable. Except in outspoken cases of gastro-intestinal pathology, however, the postprandial serum glucose concentration will not be greatly affected by absorption rates since only the *degree* of hyperglycemia is important. On the other hand, the variability in absorption will affect the oral glu cose tolerance since both *degree* and *duration* of hyperglycemia are con sidered diagnostic. With the intravenous test, however, glucose is introduced directly into the blood stream and all of the variable factors of absorption are eliminated. Since the postprandial glucose level is more convenient and simple to perform in both children and adults, it is recommended by the authors in preference to a glucose tolerance test for excluding diabetes mellitus. Special considerations may indicate the desirability of a glucose tolerance.

Postprandial Blood Sugar

Probably over 95 per cent of the glucose tolerance tests performed are done in an effort to establish or eliminate the diagnosis of diabetes mellitus; the value of the postprandial blood glucose for this purpose has been firmly established. Sindoni originally demonstrated that the concentration of glucose in blood drawn one to two hours after a meal

is rarely, if ever, elevated in normal individuals, in contradistinction to the high values found in diabetics. A number of reports have confirmed the observations of Sindoni and, in addition, have demonstrated that the postprandial blood glucose level was as reliable in diagnosing diabetes as the various modifications of the oral and intravenous glucose tolerance tests. Our experience has been similar. We have found that when blood was drawn one to two hours after a meal, the serum glucose level was always lower than 120 mg. per 100 ml. in normals and always higher than 150 mg. per 100 ml. in diabetics. The advantages to the patient of a postprandial blood sugar, rather than a glucose tolerance test, are multiple. Thus, in this procedure, the test is accomplished without fasting, is completed in a relatively short time, and requires only one rather than five or six blood samples. Furthermore, the cost to the patient is considerably reduced.

Preparation of Patient. The previous diet has a profound influence on the type of response obtained with any type of carbohydrate tolerance test. Starvation or a high fat diet results in an exaggerated elevation and a delay in the return of the blood sugar to normal. Regardless of the type of tolerance procedure to be performed, the patient should be placed on a high carbohydrate diet for at least 3 days prior to the test so that it may be carried out under optimum, standard conditions.

Procedure. The patient eats a normal, hearty meal which can be breakfast, lunch or dinner. One to two hours after eating, blood is obtained for a glucose estimation. *Excess* physical activity should be avoided during this period since muscular activity will accelerate the rate at which glucose enters muscle cells.

Interpretation. A postprandial serum glucose level of less than 140 mg. per 100 ml. is considered a normal response while one that exceeds 140 mg. per 100 ml. is classified as abnormal. The postprandial glucose level is sensitive enough to detect individuals with mild diabetes, i.e., with a moderate, persistent elevation of the blood glucose concentration but with no glycosuria. Since treatment of these patients will not include insulin but will be directed along the lines of weight reduction, if indicated, and other general measures, it is immaterial whether diabetes is diagnosed by means of a postprandial blood sugar or by means of a glucose tolerance test.

Indication for Test. Since many patients with diabetes may exhibit only minor symptoms and may not excrete sugar in the urine, it is wise to screen routinely all hospital patients for this disease by performing a postprandial blood sugar analysis as part of their work-up. It is

also of value to determine a postprandial blood sugar at regular intervals in following the course of a mild diabetic. Increased activity of the adrenal cortex, anterior pituitary, or thyroid does not always produce a hyperglycemia. With the finding of hyperglycemia, however, an investigation of these endocrine glands for hyperfunction may be indicated. The finding of reducing substances in the urine (mellituria) should be followed by a postprandial blood glucose. The finding of a normal postprandial blood sugar eliminates the diabetic syndrome as the cause. Then the possibility of the presence of renal glycosuria, fructosuria, pentosuria or lactosuria should be investigated.

Intravenous Glucose Tolerance Test

Preparation of Patients. For both the oral and intravenous tolerance test, the antecedent dietary conditions given under postprandial blood sugar should be followed. In addition, the patient must have eaten no food for at least 12 hours.

Procedure. After a fasting, venous blood sugar is obtained, $\frac{1}{3}$ gm. of glucose per kg. body weight as a 50 per cent glucose solution is injected intravenously over a 3 to 5 minute period (Soskin and Levine). This dosage is recommended for both adults and children. Subsequent blood samples are taken at $\frac{1}{2}$, 1, 2 and 3 hours after the injection. When obtained, the bloods should be sent promptly to the laboratory in order to avoid the glycolysis that occurs with standing. Although some laboratories inject a total of 25 gm. of glucose, regardless of the weight of the patient, the authors feel that more information may be obtained by employing the glucose dose of $\frac{1}{3}$ gm. per kg.

Interpretation. Three distinct types of blood sugar curves are shown in Figure 7.7. The concentration of blood glucose reaches a peak immediately upon completion of the injection, and then falls progressively. The maximum height to which the sugar rises has no significance, but the duration of the hyperglycemia is clinically important. With the $\frac{1}{3}$ gm. per kg. dose, the serum glucose normally returns to within 5 mg. per 100 ml. of its control level (70 to 105 mg. per 100 ml.) in about one hour, and may continue to fall for about another half hour to a value some 5 to 15 mg. per 100 ml. below this (see glucose section for whole blood values). Following this dip, the blood glucose concentration returns to approximately its control level. This "hypoglycemic" phase of the glucose tolerance is caused by a lag in the ability of the liver to change from glycogenic activity, resulting from the hyperglycemia, to its usual role of supplying blood glucose. If the flat dose of 25 gm. is injected the return of the blood sugar to normal may be delayed in individuals of

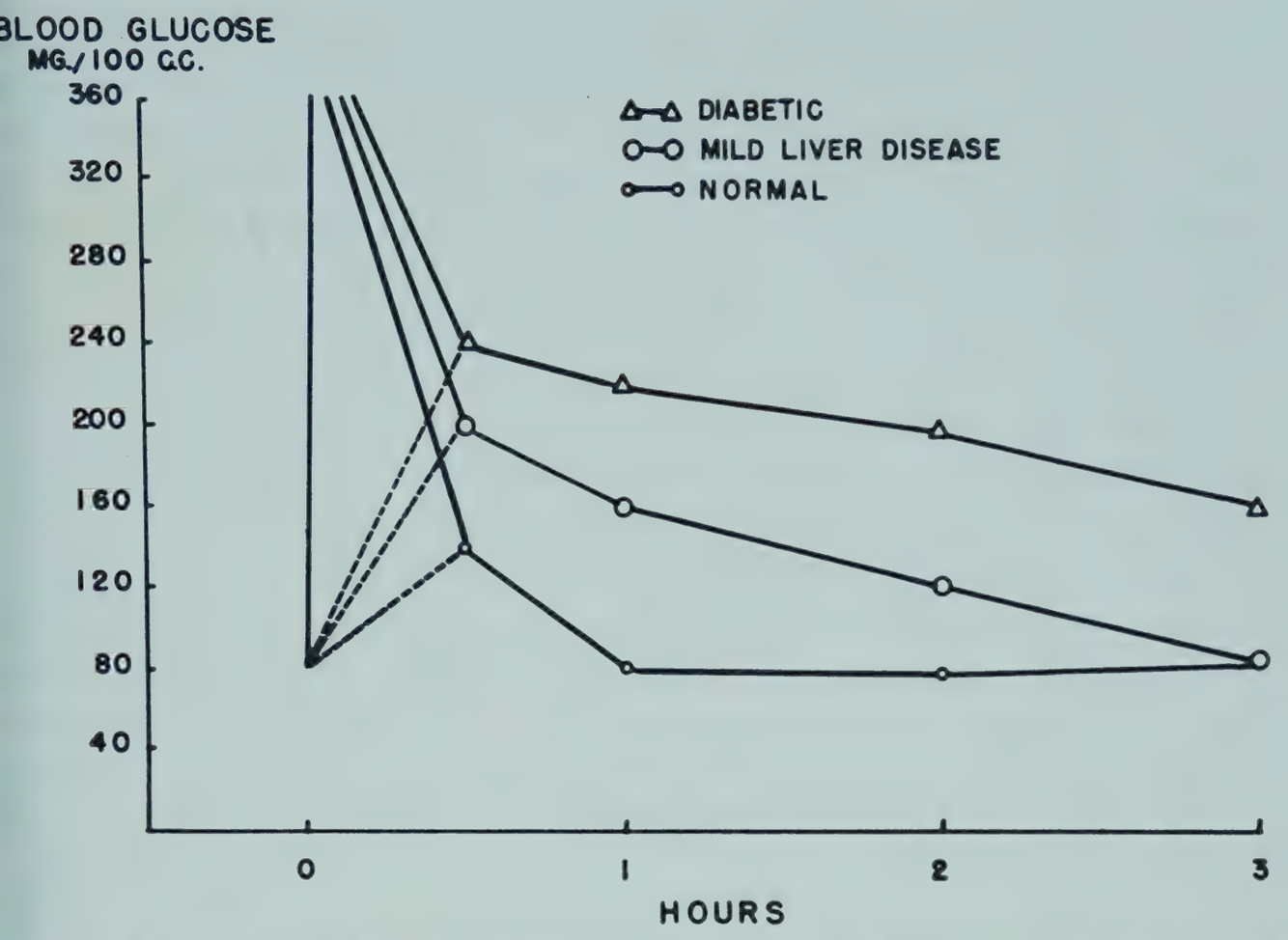


FIG. 7.7. The blood sugar values resulting from the intravenous injection of $\frac{1}{3}$ gm. of glucose per kg. into (a) a normal individual, (b) a patient with mild diabetes, (c) a patient with mild hepatic dysfunction. It will be noted that the immediate post injection values for glucose are *all* extremely elevated; the specimen obtained $\frac{1}{2}$ hour after the glucose injection is on the *down-swing* of the tolerance and if connected to the fasting value (broken lines) gives a distorted picture of the true state of the dynamics involved.

low weight but all persons without diabetes will show a normal blood sugar within 2 hours (Lozner et al.). No significant information can be derived from the sample obtained at the first half-hour interval except in some types of hypoglycemia.

Three types of abnormal response of blood sugar may be obtained after the $\frac{1}{3}$ gm. per kg. dosage. These are: (1) a delay of 3 hours or more before the blood glucose returns to normal levels; (2) a return to normal levels between one and 2 hours after the injection; (3) an exaggerated hypoglycemic phase.

The first type of curve is typical of the *diabetic syndrome.* An individual with mild diabetes may have a normal or only slightly elevated, fasting blood glucose but will not be capable of restoring the blood sugar to the control value until three or more hours after the administration of glucose. This delay is caused by an absolute or relative insulin lack which affects hepatic homeostasis.

The second type of response is found in some cases of *liver dysfunc-*

tion. A delay in the fall of the blood sugar is also found here, but it differs from the response seen in diabetes in that the blood sugar returns to the original value between 1 and 2 hours after glucose administration. In this type of patient, the abnormally functioning liver can not respond in the normal way to the stimulus of hyperglycemia and endocrine regulation. Thus, it is possible to differentiate patients with mild diabetes from those with liver dysfunction by means of the intravenous tolerance test. The oral tolerance test is incapable of making this differentiation.

Since the administration of glucose intravenously appears to subject the regulatory mechanisms to a marked stress resulting from the great hyperglycemia produced, the test tends to exaggerate the hypoglycemic phase. As a result, intravenous glucose is well suited for demonstrating the hypoglycemias associated with *hyperinsulinism, glycogen storage disease* and *adrenal cortical* and *anterior pituitary insufficiency.* However, a blood sugar after a 12- to 18-hour fast will serve as well in demonstrating the hypoglycemia. Patients who have hypoglycemia as the basis for their fatigue exhibit some abnormal depression of blood sugar during the performance of the test.

Oral Glucose Tolerance Test

Two techniques are commonly used for the administration of glucose in the oral procedure. In one method, the glucose is administered in a single dose of 100 gm. while in the other, it is given as two 50-gm. doses 30 minutes apart. The latter test is based on the assumption that a dose of glucose which is given after a previous dose has stimulated homeostatic mechanisms, will not result in a further increase in the hyperglycemia in a normal individual; individuals with diabetes will show a further elevation in the blood sugar after the second dose. However, this test suffers from many disadvantages, the most important being the demonstration that the same type of result may be obtained whether the 100 gm. are given in one or two doses (Langner et al.). Since gastric aspiration at the end of the one-hour period has recovered an average of 50 gm. of glucose, it must be concluded that the second dose could not influence the test (Leonards and Free). In addition, it has been demonstrated that increasing amounts of ingested glucose affect the duration but not the height of the hyperglycemia obtained. Only the one dose test will be discussed below.

Procedure. After a fasting blood sugar is obtained, 100 gm. of glucose, dissolved in 300–500 ml. of orange or lemon-flavored water, are

ingested and blood sugars obtained at the same time intervals as with the intravenous test. The preparation of the patient is the same as in the latter test.

Interpretation. After 100 gm. of glucose are given orally, the height of the blood sugar usually does not exceed 150 mg. per 100 ml. and this maximum is seen $\frac{1}{2}$ to 1 hour after the glucose ingestion. Blood sugars then decline, reaching the fasting level by the 2-hour mark.

Some elevation in the fasting blood sugar concentration is abnormal. After the administration of glucose, a patient with diabetes will have a peak blood sugar of more than 170 mg. per 100 ml. and this may not be attained for $1\frac{1}{2}$ to 2 hours. The fall in blood sugar is delayed and does not return to the control level for three hours or more. Hence, both the height of the blood sugar and its duration are of importance. An abnormal degree of hyperglycemia may occur after gastrectomy since the stomach empties rapidly in this condition, causing large amounts of glucose to suddenly enter the intestine ("dumping syndrome"). This complication is avoided by using the intravenous test.

In diseases of the gastro-intestinal tract associated with interference in absorption (*sprue*, *celiac disease*, *enteritis*, *colitis*, etc.) little if any hyperglycemia may be seen. In such cases, the oral test can be used to demonstrate gastro-intestinal abnormalities but it is not superior to other tests such as the rise in plasma amino nitrogen after a casein meal or the rise in serum vitamin A after its oral administration. Only a small increase in blood sugar values will be seen in *untreated anterior pituitary* and *thyroid insufficiency*. Patients with *Addison's disease* may show a normal type of curve if replacement therapy with the electrolyte- and water-regulating hormones is adequate.

Insulin Tolerance Test

The hypoglycemia which results from the intravenous injection of insulin may be used to evaluate the homeostatic mechanisms regulating blood sugar levels. In brief, this includes the liver and the secretions of the anterior pituitary, thyroid and adrenal which act to restore the glucose concentration to normal. After the intravenous injection of 0.1 unit of insulin per kg. body weight into a normal individual, the blood glucose concentration falls to approximately 50 per cent of its control value in about 20 to 30 minutes and returns to its pre-injection value within 2 hours.

In a patient with hypopituitarism, adrenal cortical insufficiency, and in some patients with anorexia nervosa, the fall in blood glucose is ac-

centuated and the blood glucose does not return to its control level for 3 or more hours. This latter phenomenon has been termed "hypoglycemic unresponsiveness" and can be attributed to a deficiency in the mechanisms stimulating gluconeogenesis, namely, adrenal cortical steroids, growth hormone, ACTH or thyroid stimulating hormone, depending on whether the primary pathology involves the adrenal or pituitary gland. The insulin tolerance test appears to be indicated in only one situation, namely, differentiating between hypopituitarism and primary hypothyroidism. When an insulin tolerance test is performed for this purpose the patient should be given a high carbohydrate diet for several days prior to the test. In order to prevent an insulin reaction, the dose of insulin administered intravenously is reduced to 0.01–0.05 units per kg. body weight. Blood samples for glucose determination are drawn at 0, 20, 30, 45, 60, 90, and 120 minutes after the injection. The blood sugar in the patient with hypopituitarism decreases in concentration to about 50 per cent within 20 to 30 minutes and does not return to the preinjection level for more than 2 hours. In contrast to this, the blood glucose of the individual with primary hypothyroidism usually does not reach its lowest point for at least 45 minutes and the per cent drop is characteristically less than that seen in the former case.

There is no need to perform insulin sensitivity tests in individuals suspected of having adrenal or pituitary insufficiency since more specific tests exist for demonstrating the deficiencies. It may even be dangerous to attempt the test in a patient with Addison's disease. The test is of no use whatsoever in the diagnosis of hyperinsulinism since this condition is caused by hypersecretion of insulin and not by increased sensitivity to the hormone.

PROTEIN-BOUND IODINE

Iodine is important for the normal functioning of the body since it is an integral part of the hormone(s) of the thyroid gland. This element is therefore essential for the formation of an important metabolic regulatory factor, thyroxin. In plasma, the concentration of iodine bound to protein correlates well with the level of circulating thyroxin.

Physiology. (Rosenberg and Astwood; Sunderman and Sunderman; Rawson). There are approximately 10–15 mg. of iodine in the average human adult. Of this amount, less than 1 per cent is in the form of inorganic iodine and more than 80 per cent exists in organic combination in the thyroid gland. In man, on a diet containing "normal" amounts of iodine, about 150–200 micrograms of the element are ingested and a

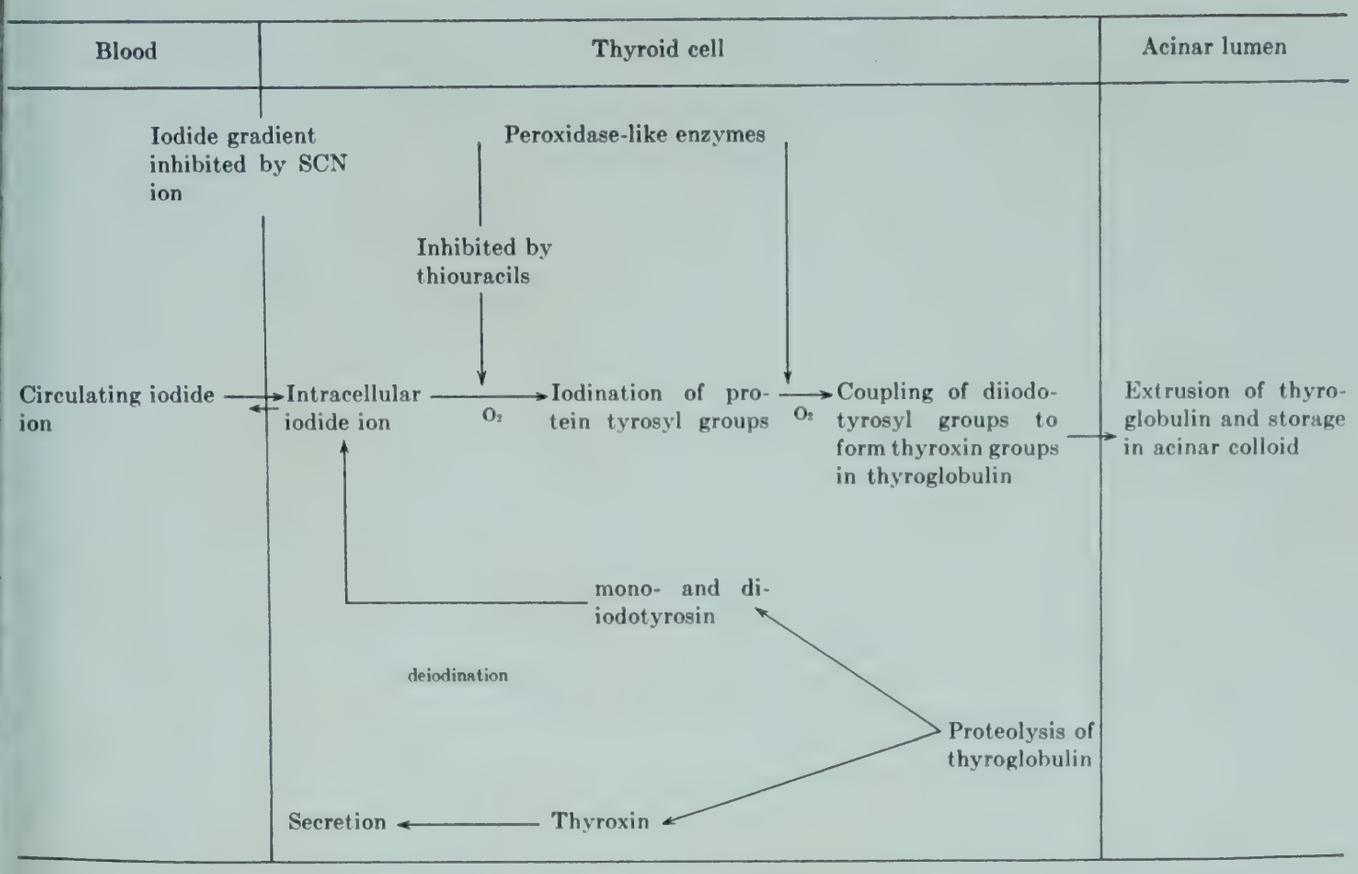


FIG. 7.8. Simplified illustration of thyroxin formation and release. (Modified from Rosenberg and Astwood.)

similar amount is excreted daily by the kidney. There is a reciprocal relationship between iodide accumulation by the thyroid and renal excretion of the ingested ion, as shown by studies with radioactive material (I^{131}).

The fate of that portion of the absorbed iodide which is utilized for the formation of thyroid hormone is shown diagrammatically in Figure 7.8. It may be seen that essentially, the following reactions occur: (a) concentration of iodide; (b) oxidation of iodide to iodine, with prompt iodination of tyrosyl derivatives to form thyroxin.

The thyroid gland is unique in its ability to concentrate and organically bind iodide ion. Although the mechanism for the process is unknown, energy is apparently required for concentrating and maintaining a gradient against plasma. The trapped inorganic iodide is thought to be oxidized to elemental iodine or hypoiodate, which in turn reacts with tyrosyl derivatives of protein to form mono- and diiodotyrosine within protein itself. Thyroxin is formed from an oxidative condensation of the iodinated tyrosyl derivatives and stored in the acinar lumen as thyroglobulin. As needed, this protein is degraded and thyroxin secreted into plasma. During the process of proteolysis, any mono- and diiodotyrosine formed is apparantly deiodinated and the iodine reutilized for thyroxin formation. Normally, approximately 360 micro-

grams of thyroxin are secreted by the thyroid daily; this may rise to ten times this amount in individuals with hyperthyroidism.

A number of conditions and factors influence the rate of thyroid hormone synthesis and release. The thyrotropic hormone of the anterior pituitary (TSH) stimulates two processes in the thyroid: (a) the accumulation of iodide, and its subsequent organic binding, the latter probably occuring as a result of the increased availability of iodide; (b) the rate of release of thyroid hormone into plasma. In the absence of the pituitary, these processes proceed at approximately 10 per cent of the normal rate.

The release of TSH, in normal individuals, is affected by the concentration of circulating thyroid hormone and tends to vary inversely with the latter. Thus, administration of exogenous thyroxin will inhibit endogenous hormone formation apparently through an effect on the pituitary. In addition to the effect of TSH per se, a number of other factors, working either through the pituitary or directly on the thyroid, influence the rate of thyroxin formation. These factors include (a) environmental temperature, with which the rate of thyroxin formation tends to vary inversely, (b) adrenal cortical and androgenic gonadal steroids, which in excess may decrease thyroid hormone formation, the former by inhibiting TSH effects on the thyroid, (c) goitrogenic diets (see Figure 7.8), which interfere with thyroxin formation, although the balance between intakes of goitrogenic factors and iodide will determine whether inhibition occurs or not, (d) inorganic iodide ingestion in excess which has an inhibitory effect on organic iodine binding by the thyroid. In addition, it has been suggested that inorganic iodide (a) inactivates thyrotropin and thus interferes with TSH action on the thyroid and, (b) inhibits the proteolytic enzyme which liberates thyroxin from thyroglobulin.

The hormone(s) of the thyroid is thought to be thyroxin (and/or triiodothyronine); the hormone has a profound effect on growth, organic metabolism and calorigenesis. Thyroxin has a half life in the human of between 7 and 12 days. It is carried in plasma by a protein migrating electrophoretically between the alpha-1 and alpha-2 globulins.

Iodine in serum exists in both the inorganic and organic state, the latter comprising approximately 90 per cent of the total serum iodide and consisting almost entirely (90 per cent) of thyroxin. Almost all the organic iodide is precipitated with the serum proteins (protein-bound iodine, PBI) by the common protein precipitants. PBI values exceed thyroxin iodine values by about 0.6 micrograms per 100 ml. because of the presence of small amounts of triiodothyronine or other organic iodi-

nated compounds in serum. Thyroxin iodine per se can be determined as the non-alkali soluble fraction of the butanol extractable portion of serum iodide.

Normal Concentration. The mean value for serum PBI in normal adults is considered to be 5.0 micrograms per 100 ml., with a range of 3.0 to 8.0 micrograms per 100 ml. In new-born infants, the mean value is 8.3 micrograms but rises to 12 micrograms per 100 ml. early in the first week of life. After seven days, serum PBI levels slowly decline to a mean value of 6.3 micrograms per 100 ml. by the end of the first year. Children above the age of 3 years have serum PBI levels that approximate those of adults. Serum PBI has been reported to decrease slightly after the age of 45 in men but not in women. There is normally an increase in the concentration of PBI during pregnancy, starting as early as the third to sixth week after conception, so that mean values of approximately 8 micrograms per 100 ml. have been reported. By the 5th to 8th week following parturition, the mean PBI values are somewhat lower than normal non-pregnant females and continue thus for about a year. Long standing acclimatization to low environmental temperatures may be associated with increased PBI concentrations.

Increased Concentration. Since protein-bound iodine concentrations reflect the level of circulating thyroid hormone, PBI levels are a sensitive index of thyroid function. Increased values are most commonly seen in *hyperthyroidism*. When the results of numerous investigators are compiled, more than 94 per cent of patients with hyperthyroidism have serum PBI values exceeding 8 micrograms per 100 ml. In general, it was found that serum PBI levels were more consistently elevated in individuals with frank thyrotoxicosis than in those in whom the clinical picture was less well marked. It should be emphasized that there is an area of overlap in the PBI levels of normal and hyperthyroid individuals, the amount of overlap varying with the different reports. In addition to hyperthyroidism, an elevated PBI may be seen in *acromegaly* with hyperthyroidism, during the *first 24 hours after thyroidectomy*, and in *early hepatitis*. Values in hepatitis return to normal within a few weeks.

Decreased Concentration. Although serum PBI values of less than 3 micrograms per 100 ml. have been obtained in over 98 per cent of the patients with hypothyroidism that have been reported, some overlap of PBI levels of patients with hypothyroidism and normal individuals does occur, however.

Myxedema may result from primary thyroid dysfunction or be second-

ary to lack of pituitary TSH, caused by either panhypopituitarism or by a unitropic deficiency of the thyroid stimulating hormone. Patients with *nephrosis* may also exhibit low serum PBI concentrations but this finding may reflect a decrease in the thyroxin-carrying protein. As mentioned previously, *ACTH* and *cortisone* administration in therapeutic doses may sometimes depress serum PBI values.

Indication for Test. As with other tests of thyroid function, serum PBI levels do not always yield results which are consistent with clinical findings. In both hypo- and hyperthyroid individuals, at both extremes of the range of normal, there is an overlap into the normal area. This overlap has been attributed among other things, to the large variability of the level of circulating thyroid hormone in the normal population. A random specimen on any individual yields no information as to what PBI level was normal for him nor any changes from this level that might have occurred with time. A number of precautions must be observed if the determination and interpretation of serum PBI values are to be free of errors. (1) The chemical determination must be performed in a laboratory free of iodine and its vapors. (2) The administration of thyroid extract or thyroxin must be avoided since they will increase serum PBI levels; this could be interpreted as indicating hyperthyroidism. (3) Iodine-labelled X-ray contrast media should not be adminstered prior to the determination of a PBI since the serum organic iodide fraction may remain elevated from days to years following their use. (4) The use of iodine or thiourea compounds *may* depress PBI levels in hyperthyroid individuals and yield values which cannot be correctly interpreted. On the other hand iodide administration may also elevate the serum organic iodide fraction. Under such circumstances serum thyroxin determinations may be of greater diagnostic value. (5) Mercurial diuretics may lower PBI levels for 24 hours after administration.

The determination of PBI is best suited for individuals in whom the clinical picture and other laboratory procedures (BMR, thyroidal I^{131} uptake) are equivocal and those in whom it is not possible to obtain a satisfactory BMR. Patients with cardiac failure, chest disease, mental and neurologic disease and children suspected of thyroid dysfunction fall into this latter category. In addition, disease states associated with hypermetabolism but not hyperthyroidism may be differentiated, such as leukemias, Hodgkin's disease and polycythemia vera.

Method (Barker and Humphrey). An aliquot of serum is treated with a protein precipitant, the precipitate of protein and iodine centrifuged, washed, alkali digested and redissolved. The iodine in the solution is

determined by its ability to catalytically reduce ceric ion thus

$$\underset{\text{(yellow)}}{\text{Ceric sulfate}} + \text{arsenious acid} \xrightarrow{I^-} \underset{\text{(colorless)}}{\text{Cerous sulfate}}$$

Volume of serum: 2 ml.

ELECTROLYTES

Sodium, potassium, chloride and bicarbonate are the electrolytes mainly concerned with the movements of body water and the maintenance of normal acid-base relationships in intra- and extracellular fluids. As can be seen in Table 7.4, the total milliequivalents of cations and anions are equal.

Utilizing the method of expressing concentrations as milliequivalents per liter, changes in the concentration of an ion can be evaluated with regard to its effect on fluid balance and exchange and pH disturbances. The older method of expressing concentration in mg. per 100 ml. obscured this information. In the discussion that follows, electrolytes are discussed in terms of milliequivalents and bicarbonate in terms of millimoles per liter. The conversion of m.e./L and mM/L to mg./100 ml. and volume per cent, respectively, are given by the following formulas:

$$\text{mg./100 ml.} = \text{m.e./L} \times \frac{\text{equivalent weight}}{10}$$

$$\text{volume } \% = \text{m.M./L} \times 2.24$$

TABLE 7.4

*Concentration of Cations and Anions in Plasma and Cell Water**

Cation	Concentration (m.e./L.)		Anion	Concentration (m.e./L.)	
	In plasma water	In cell water		In plasma water	In cell water
Sodium	154	15	Bicarbonate	29	10
Potassium	5	150	Chloride	111	1
Calcium	5.4	2	Phosphate	2	97
Magnesium	2.6	15	Sulfate	1	15
			Organic acid	7	—
			Proteinate	17	59
Total	167	182		167	182

* The intracellular concentrations given for the electrolytes are only approximate since the values are dependent upon indirect measurements.

The equivalent weight of a substance is the weight in grams that will displace 1 gram mole of hydrogen or hydroxyl ion from chemical combination; a milliequivalent is $\frac{1}{1000}$ of this amount.

Body Water

A description of the handling of electrolytes by the body is complicated by the fact that it entails a concurrent study of body water and shifts of electrolytes and water into and out of cells. Before considering the individual electrolytes, therefore, it appears desirable to briefly discuss water and its distribution (Edelman, Haley, et al.).

Water is the most important constituent of the body from the standpoint of percentage composition (vide infra) and is found both inside and outside of cells. Extracellular fluid consists of the water found in both the vascular channels and interstitial spaces while, intracellular water refers to that portion within cells. "Transcellular water"—cerebrospinal fluid, gastrointestinal secretions—is considered as a portion of the extracellular body water which is not measured by the usual techniques. Although for physiological and clinical purposes it is useful to describe water as being confined to the inside or outside of the cell, the connotations of a rigid barrier at the cell wall are not correct. Water is *constantly and rapidly* passing back and forth between all "compartments" of the body and exchanging through capillary and cellular membranes; a state of dynamic equilibrium exists.

Infants and children contain a higher percentage of body water than adults. During the first month of life, approximately 76 per cent of the body weight is water, but this drops to 63 per cent by the ninth month. The relative water content of children continues to decrease gradually with age, reaching average values for males of 61 per cent of the body weight and 51 per cent for females by the age of 17 years. The slow decline of the per cent of body water continues with age. A portion of this decline may be related to an increase in body fat since the deposition of fat is accompanied by very little water (0.1 gm. water per gm. fat in contrast to approximately 4 gm. water per gm. protein). Extracellular fluid in the infant is approximately 48 per cent of the body weight; with maturity, this compartment decreases to about 15 per cent of body weight in males and 14 per cent in females.

A 70-kg. adult, with 42 liters of body water, loses approximately 6 per cent of this water daily through urination, respiration, perspiration and defecation. For an individual in water balance, this loss is met primarily by drinking fluids; a smaller amount is contributed by water

contained in foods and arising from oxidation in the body. In contrast to adults, a 10-kg. child, with only 6 liters of body water, turns over 15 per cent of his total fluid each day. This latter fact explains the relative rapidity with which children are prone to become dehydrated if their daily losses are not replaced.

Within the limits of this chapter, it is not possible to discuss all the aberrations in the distribution of water that occur in disease. Moreover, a description of the movement of water of necessity requires a description of movements of electrolytes and will be described in subsequent sections. Edema and dehydration are terms commonly employed to describe the state of increased and decreased extracellular fluid volumes, respectively, as compared to normal. In the sections that follow, it should be born in mind that extracellular edema may occur with either an intracellular edema or dehydration and the same is true for extracellular dehydration. The state of hydration of the cell is *not* necessarily correlated with the state of extracellular fluid volume.

Bicarbonate and pH

Metabolism. The hydrogen ion concentration of serum and other extracellular fluids is maintained within a narrow normal range by a series of buffer systems, the most important of which is the bicarbonate/carbonic acid ($[BHCO_3]/[H_2CO_3]$) system. This careful regulation of the environment is necessary for normal cellular activities, for even with relatively small changes in hydrogen ion concentration, death of cells occurs. Lesser changes are probably associated with some impairment in tissue functions.

The term pH has been introduced to simplify the designation of the hydrogen ion concentration of aqueous solutions. By definition, $pH = \log \frac{1}{[H^+]}$. The pH of a neutral solution is 7.0 while that of blood is approximately 7.4. It should be noted that this pH is slightly on the alkaline side of neutrality, and that the terms acidosis and alkalosis as employed medically are really relative. Since bicarbonate is so intimately concerned with the regulation of serum pH, the two subjects will be discussed together.

Buffer Systems (Peters and Van Slyke, 1931; Gamble). The buffers of the blood are the salts of bicarbonate, hemoglobin, phosphate and plasma proteins. These are all salts of weak acids and efficiently resist changes in pH. Normally about 65 per cent of the buffering capacity of blood is accomplished by the bicarbonate, about 30 per cent by the

hemoglobin and the remaining 5 per cent by the protein and phosphate systems.

Since the buffer systems are in equilibrium with each other, the state of bicarbonate will reflect the state of the other buffers. Bicarbonate is the most flexible anion with respect to variations in serum concentration because, under ordinary circumstances, there is virtually no limit to its production in tissue cells and to its excretion by the lungs. Since sodium comprises more than 90 per cent of the total cations, the amount of sodium unassociated with other anions is the factor that determines the bicarbonate concentration. This is indicated by the equation below which follows from the fact that the sum of the cation concentrations must equal the sum of the anion concentrations:

$$[HCO_3^-] = ([Na^+] + [K^+] + [Ca^{++}] + [Mg^{++}]) - ([Cl^-] + [HPO_4^{--}] + [HSO_4^-] + [\text{Organic acids}^-] + [\text{Proteinate}^-])$$

For practical purposes, the pH of serum can be calculated from the Henderson-Hasselbalch equation which states that $pH = 6.1 + \log [BHCO_3]/[H_2CO_3]$. As can be seen from the equation, the important factor governing pH is the ratio of bicarbonate to carbonic acid; this ratio is normally 20 to 1 (27 mM/L as $BHCO_3$ and 1.35 mM/L as H_2CO_3).

A number of acids are formed during cellular metabolism, most of which are neutralized by bicarbonate and other buffers of extracellular fluids. Some of these acids, such as lactic, citric, pyruvic and a portion of the carbonic, may appear in the blood during their transport to other organs for participation in metabolic processes. As shown in Table 7.4, these constitute only a minor portion of the anions. The main acid produced, carbonic acid, is volatile while others—phosphoric and sulfuric—are non-volatile. The capacity of the buffer systems to regulate serum pH is maintained by the combined activities of lung and kidney. Carbonic acid is eliminated by the lungs through the volatilization of CO_2 while the two above mentioned non-volatile acids, together with chloride, are excreted by the kidney. During periods of alkalosis, the kidney may also excrete bicarbonate until the acid-base balance is restored to normal.

Role of the lungs in the maintenance of bicarbonate and pH: As CO_2 is produced in tissue cells during the course of catabolic processes, most of it diffuses into the blood capillaries. It is rapidly converted in red cells to carbonic acid through the catalytic action of the enzyme, carbonic anhydrase. Approximately 90 per cent of the H_2CO_3 formed is neutral-

ized according to the reaction*: $H_2CO_3 + KHbO_2 = KHCO_3 + HHb + O_2$. The bicarbonate formed diffuses into plasma while chloride replaces it in the cell. Despite the fact that both $BHCO_3$ and H_2CO_3 are increased in amounts, there is little change in the pH since the 20:1 ratio of $BHCO_3$:H_2CO_3 is maintained by the incomplete neutralization of H_2CO_3.

The above reactions are reversed in the lungs where volatile CO_2 is eliminated by exhalation and the normal ratio $BHCO_3$:H_2CO_3 is not disturbed. The lungs control the concentration of carbonic acid by means of the depth and rate of respiration. Increased respiratory exchange blows off an excess of carbonic acid and unless renal adjustments are made, the ratio of $BHCO_3$:H_2CO_3 increases and an alkalosis results. Decreased pulmonary exchange will have the opposite effect.

Role of the kidneys in the maintenance of bicarbonate and pH: In the course of urine formation, the kidneys play an important role in maintaining constant the internal environment of the body. This is accomplished by the ability of the renal tubules to regulate the output of water and electrolytes by variations in processes of reabsorption and/or secretion. The excretion of non-volatile acids would soon deplete the body of its sodium and potassium and result in acidosis but for renal tubular activities which serve to conserve body electrolytes. Base is saved in the body by the ability of the renal tubules to exchange H^+ for Na^+ during the excretion of these acids, as follows: $Na_2HPO_4 + H_2CO_3 = NaH_2PO_4 + NaHCO_3$. Thus the acid salts are excreted and the bicarbonate is reabsorbed; base is saved by the excretion of urine with a pH lower than that of blood. In severe acidosis, base is lost when acid production exceeds the capacity of the renal tubules to exchange H^+. Conversely, when chloride depletion and alkalosis exist, the administration of NaCl and KCl results in the retention of chloride by the kidney and the excretion of the excess Na^+ in the urine as $NaHCO_3$.

Another mechanism that conserves base is the capacity of the kidney to form ammonia from glutamine and the ability to excrete ammonium salts instead of salts of fixed base. Thus, the kidney also controls bicarbonate concentration by regulating acid excretion in conjunction with that of ammonia in urine.

Normal Concentration. The serum pH of normal persons lies between 7.35 and 7.45. The CO_2 capacity (combining power) varies between 25 and 31 mM/L (56 to 70 volumes per cent). There is a slight decrease

* HHb is an abbreviation for the acid of reduced hemoglobin while $KHb0_2$ denotes the potassium salt of oxidized hemoglobin.

in serum CO_2 capacity during the latter months of pregnancy. For a short time after birth, infants likewise show some reduction in CO_2 capacity.

Abnormal Concentration. The finding of a low CO_2 capacity in serum has usually been designated as an acidosis and a high one as an alkalosis. True acidosis or alkalosis, however, depends upon a change of pH and therefore upon an alteration in the ratio $BHCO_3:H_2CO_3$. If the ratio remains normal, no change in pH will occur regardless of the absolute value of the CO_2 capacity. A disturbance in acid-base balance may occur under various conditions (see Table 7.5). The principal ones are:

1. *Primary CO_2 excess:* With hypoventilation, such as may be seen in *central nervous system depression* or certain *pulmonary diseases*, the value of the ratio $BHCO_3:H_2CO_3$ decreases below its normal value of 20:1. A relative increase in carbonic acid takes place as the lungs become unable to expel sufficient amounts of CO_2. There is some attempt on the part of the kidney to adjust to this situation by an increased excretion of chloride, making more sodium available for bicarbonate. Usually this adjustment is not perfect so that despite an increase in CO_2 capacity, the pH of serum is less than 7.35. *Under these conditions, the determination of a CO_2 content or capacity will be entirely misleading.*

2. *Primary CO_2 deficit:* With hyperventilation, as may occur with increased *central nervous system excitation*, *hysteria* and *fever*, an excess of carbonic acid is blown off through the lungs so that there is a relative carbonic acid deficiency as compared to the bicarbonate. The renal adjustment consists of a loss of bicarbonate in the urine while retaining chloride in the body. Although an immediate determination may yield a normal serum CO_2 capacity, as adjustments occur, the CO_2 capacity declines. *Again, the conditions are such that a CO_2 determination alone leads to misleading information since the serum pH is above 7.45.*

3. *Primary alkali deficit:* A deficit in alkali, either absolute or relative, may cause a decrease in both serum pH and CO_2 capacity. This may be produced in several ways. With excessive loss of base, as may occur in *diarrhea*, *Addison's disease*, or *intestinal fistula*, there is a decrease in the sodium available for combination with bicarbonate and the ratio, $BHCO_3:H_2CO_3$ acid is disturbed; an acidosis is produced. In some types of renal tubular insufficiencies, as in the *Fanconi syndrome*,* the

* *Fanconi Syndrome:* A symptom-complex of unknown etiology which occurs chiefly in infants and children. It is characterized by various combinations of glycosuria, hyperaminoaciduria, hypophosphatemia, some decrease in serum CO_2

kidney may lose excessive amounts of base. In *bilateral renal disease* tubular involvement may result in a diminished ability to form ammonia and exchange H^+, resulting in excessive loss of base. Furthermore, since the capacity to excrete phosphate and sulfate is decreased, the increased serum levels of these acids render less sodium available for combination with bicarbonate. All factors operate to reduce the pH and CO_2 capacity. Excessive production of organic acids causes loss of base as sodium or potassium salts through urinary excretion. These acids also displace $BHCO_3$ in serum. Thus in *diabetic acidosis*, the excessive production of acetoacetic and betahydroxybutyric acids are responsible for the low pH and CO_2 capacity of serum. Some degree of compensation will result from the increased respiratory rate which may be present and which tends to lower the carbonic acid content. During treatment of the acidosis, it is important that pH determinations be performed if alkali is administered as part of the therapy. If reliance is placed solely on the CO_2 capacity, too much alkali may be given. The ratio of $BHCO_3$:H_2CO_3 may be restored to normal (with a normal pH) while the CO_2 capacity remains low.

4. *Primary alkali excess:* With *pyloric* and *high intestinal obstruction*, there is a loss of hydrochloric acid from the stomach. In the serum, this is manifested by a decrease in chloride, making more base available for combination with bicarbonate. As a result, there is a change in the $BHCO_3$:H_2CO_3 ratio and both the CO_2 capacity and pH are indicative of an alkalosis. However, if there is an achlorhydria or if the vomiting persists for several days, the starvation attending the vomiting leads to excessive production of organic acids and an acidosis may be seen. An alkalosis may also result from excessive loss of gastric contents through *excessive Wangensteen drainage* of the stomach. *Cushing's syndrome* and *excessive administration* of *ACTH* or *cortisone* may likewise produce an alkalosis since KCl in excess is lost in the urine (see potassium section).

Indication for Tests. Since deviations of pH from normal are associated with morbidity, this determination is often of more value than the measurement of the CO_2 capacity, which can even be misleading. This is especially true in pulmonary disease and in illness in which there are changes in the depth or rate of respiration. Both determinations, of

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capacity and some elevation in alkaline phosphatase activity. Serum urea levels are normal. Pathologically, rachitic bone changes and cirrhotic changes in the liver have been described. Some features of the syndrome appear to be explainable on the basis of altered renal tubular transfer mechanisms. The causes of the bone and liver pathology are unknown.

course, are more meaningful than either alone and should be done simultaneously, if possible. Thus, pH and CO_2 values are indicated whenever a disturbance in acid-base balance is suspected. For intelligent management of derangements, determinations of sodium, potassium and chloride may also be advisable.

Method. *pH.* Blood for a pH determination is collected under oil. The pH is measured directly by means of a glass electrode or colorimetrically by the use of phenol red as an indicator (Van Slyke et al. 1949).

Volume of Serum. 0.05 ml. (micro glass electrode) or 0.5 ml. (Van Slyke method).

CO_2 Capacity (Van Slyke and Cullen, 1917; Natelson). Serum is equilibrated with alveolar air and the CO_2 measured gasometrically in a Natelson or Van Slyke apparatus.

Volume of Serum. 0.03 ml. (Natelson) or 1 ml. (Van Slyke).

TABLE 7.5

*Serum Electrolyte Values in Conditions Characterized by Acid-Base Imbalance**

Disease	Serum Concentration				
	pH	Sodium	Potassium	Chloride	CO_2 capacity
Primary CO_2 excess (respiratory acidosis; hypoventilation).....	↓	→	→ ↑	↓ →	↑ →
1. Respiratory center depression: drugs and injury					
2. Paralysis of respiratory muscles					
3. Pulmonary disease: emphysema, bronchiectasis, pneumothorax, pulmonary edema					
4. Congestive heart failure (some cases).....		↓ → ↑			
Primary CO_2 deficit (respiratory alkalosis; hyperventilation).....	↑	→	↓	↑ →	↓ →
1. Respiratory center stimulation					
a. C.N.S. disease					
b. Drugs—salicylates					
2. Hysteria and anxiety					
3. Fever					
4. High altitudes					

TABLE 7.5—*Continued*

Disease	Serum Concentration				
	pH	Sodium	Potassium	Chloride	CO_2 capacity
Primary alkali deficit (metabolic acidosis)	↓ →	↓	↑ →	↓	↓
1. Excessive loss of base					
a. Diarrhea, Addison's disease					
b. Renal disease					
Renal insufficiency	↓ →	↓ → ↑	→ ↑	↓ →	↓ →
Renal tubular insufficiency	↓ →	→	↓	↑	↓
Fanconi syndrome	↓ →	→	→ ↓	↓	↓
Milkman's syndrome	↓ →	→	→ ↓	↓	↓
2. Excessive production of organic acids	↓	↓	↑	↓	↓
a. Disturbed food intake: starvation, high fat diet, vomiting (late)					
b. Endocrinopathies, chiefly diabetes mellitus					
c. Impaired liver function: hepatitis, glycogen storage disease					
d. Violent exercise	→	→	→ ↓	→	↓
e. Administration of acids: calcium chloride, ammonium chloride, hydrochloric acid	↓	↓	→	→ ↓	↓
Primary alkali excess (metabolic alkalosis)					
1. Excessive loss of acid: pyloric or high intestinal obstruction (early), gastric suction, diarrhea of pancreatic fibrosis	↑ →	→ ↑ ↓	→ ↓	↓	↑
2. Body potassium deficit: poor intake, Cushing's syndrome, intestinal fistulas, prolonged treatment with I.V. glucose and saline	↑ →	→	→ ↓	↓	↑ →

* ↑ = increased values; → = normal values; ↓ = low values.

Chloride

The chloride ion comprises approximately 65 percent of the total extracellular anions. For this reason, chloride plays a major role in acid-base relationships and indirectly, perhaps, is a factor in the maintenance of body water. Of all the anions, it is the one in lowest concentration in intracellular fluids.

Physiology (Darrow and Pratt; Peters, 1947; Peters, 1944). Chloride is ingested as a sodium, potassium, calcium or magnesium salt in the food. Usually about 2.5 grams are consumed daily and are absorbed mainly by diffusion across the intestinal mucosa. Serum chloride is in equilibrium with interstitial chloride at the capillary membrane; the concentrations of the two are practically identical, the differences being predictable on the basis of Donnan membrane equilibrium factors. A normal 70-kg. adult has approximately 85 gm. (2400 m.e.) in his body whereas a 10-kg. child contains 15 gm. (420 m.e.). Under normal conditions, about 4 per cent of the body chloride of both adult and child are turned over daily.

Except for the fact that little if any chloride is found in intracellular fluid, the handling of sodium and of chloride in the body are closely related. The same physical, chemical and endocrine factors that influence sodium distribution also affect chloride. Serum chloride concentration often varies inversely with that of $BHCO_3$, the prime factor governing pH. As a result, elevations in chloride are usually accompanied by an acidosis and decreased concentrations associated with an alkalosis. Excess of some adrenal steroids will cause a decrease in the serum concentration as well as the total body content of chloride.

Most of the chloride lost from the body is excreted in the urine, in combination with sodium, potassium or calcium. Small amounts are also normally lost in the insensible perspiration. With increased amounts of sweating, this route of loss must be considered. As with sodium, the kidney can vary the proportions of water and chloride excreted in the urine and so aid in the maintenance of normal amounts and concentrations in the body.

Normal Concentration. Serum chloride is present at a concentration of 100 to 106 m.e./L. The same precautions used in interpreting serum sodium concentrations should be used with regard to the meaning of a serum chloride level, since chloride and water can vary independently of each other. Therefore, hematocrit values, body weight, and the clinical state and history must be properly evaluated.

Increased Concentration. With the exception of a pure water de-

ficiency (see Sodium section), most cases of hyperchloremia result from a primary CO_2 deficit (see bicarbonate and pH section). This type of alkalosis, which is respiratory in origin, results from hyperventilation. The increased respiratory activity can result from *drugs, disease processes which stimulate the "respiratory center," hysteria, anxiety, fever* and from the *decreased oxygen tension* as found at high altitudes. With loss of CO_2 through the lungs, some degree of compensation is achieved by renal retention of chloride which in turn displaces $BHCO_3$. Some types of renal tubular disease with acidosis likewise may exhibit an increase in serum chloride concentration. An excessive intake of NH_4Cl, leading to acidosis, or of KCl may be followed by high serum chloride levels.

Decreased Concentration. The diseases in which abnormalities of serum chloride can be expected are shown in Table 7.5. Patients with hypoventilation caused by *depression of the central nervous system, paralysis of the respiratory muscles* and *pulmonary disease* have a primary CO_2 excess which produces a hypochloremia. The mechanisms involved are the opposite of those described for hyperventilation. With depressed respiration, chloride is apparently lost in the urine in combination with ammonium or potassium, thus leaving more sodium available for combination with bicarbonate.

Chloride may be lost in the urine in *chronic renal disease*, either because of extracellular displacement by the retained sulfate and phosphate or by some tubular defect which allows for an excess urinary excretion. In *uncontrolled diabetes mellitus*, the concentration of serum chloride decreases because of the lowered total base; with a concomitant decrease in extracellular fluid volume, total body chloride is lowered. The same type of disturbance in body chloride may occur in *starving individuals* or those on a *high fat diet;* chloride is displaced by keto-acids when overproduction of the latter results in an increase in their serum concentration. Decreased concentrations of chloride are seen with both *hypo-* and *hyperfunction of the adrenal cortex.* With adrenal cortical insufficiency, chloride, together with sodium, is lost in the urine resulting in an acidosis. With adrenal cortical hyperfunction and after the administration of an excess of some adrenal steroids or ACTH, chloride likewise is lost from the body; under these conditions it is excreted in combination with potassium. Since sodium is retained and $BHCO_3$ increased at the expense of excreted chloride, this deficiency of chloride results in a metabolic alkalosis.

The excessive loss of intestinal secretions may cause a hypochloremia, but the degree depends upon the site and extent of the loss. Since

gastric secretions are high in chloride and low in base, vomiting caused by pyloric stenosis or high intestinal obstruction produces a deficit in body chloride. Fistulas of the upper gastro-intestinal tract may also produce a hypochloremia since the drainage may contain the high chloride content of the gastric secretions. Diarrhea can lead to a hypochloremia or even a hyperchloremia, depending upon the amount of water lost in conjunction with the electrolyte.

Indication for Test. Serum chloride determinations should be performed whenever a disturbance in water and electrolyte metabolism or acid-base balance is suspected (see sodium, potassium and bicarbonate sections). Serial values are helpful in estimating the stores of this electrolyte in patients who are receiving parenteral fluids. Since overdosage with both ACTH and cortisone produces a hypochloremic alkalosis, serum chloride values can be utilized for guiding the dosage of these drugs.

Method (Van Slyke and Hiller). The serum proteins are precipitated with a sodium tungstate-phosphoric acid reagent at the same time that serum chloride is precipitated by the silver of insoluble silver iodate. The iodate, which is displaced by chloride, goes into solution and is measured by titration with sodium thiosulfate.

Volume of Serum. 0.5 ml.

Phosphorus

There are about 700 gm. of phosphorus present in the body in both inorganic and organic forms. Inorganic phosphorus *per se* is found mainly in bone in combination with calcium, with only small amounts in cells and extracellular fluids. However, large amounts of phosphorus in organic combination (Table 7.4), are found intracellularly. The organic phosphorus compounds are of three types: those involved in fat metabolism (phospholipids), those concerned with energy-linked metabolic reactions (purine, glucose, and creatine phosphates) and those associated with nucleo-proteins. The discussion that follows is concerned only with the metabolism and significance of the inorganic fraction of serum phosphorus.

Physiology (Everett; Cantarow; Albright and Reifenstein). The phosphorus of foodstuffs enters the body in both organic and inorganic form. As a result of digestive activity, organic phosphate esters are hydrolyzed and the phosphate is absorbed in the inorganic state. Studies concerning the absorption of ingested phosphate compounds are complicated by the fact that phosphorus also enter the gut in the secretions of

the intestinal mucosa. It is known, however, that the absorption of phosphorus from the gastro-intestinal tract is controlled by essentially the same factors that are involved in calcium absorption (see calcium section). The intestinal pH, the amounts of calcium and magnesium present in the diet, and the intake of vitamin D all influence the process. Furthermore, colloidal aluminum hydroxide, which is often used for the treatment of patients with peptic ulcers or with renal insufficiency, prevents the intestinal absorption of phosphate by forming insoluble aluminum phosphate. The average daily intake of phosphorus is about double that of calcium, averaging approximately 1.2 gm. If the individual is in normal phosphate balance, a similar amount is lost from the body in the urine and feces. Approximately 60 per cent of the phosphorus is excreted in the urine.

The phosphorus of serum is practically all in the inorganic form and serves as one of the buffer systems of the blood. In cells, phosphate is bound as an ester or in high energy linkage to a number of organic compounds that are metabolically active. In bone, phosphate is deposited in the matrix as an insoluble calcium salt; this phosphate is in dynamic equilibrium with that of extracellular fluid and a continual exchange of phosphorus takes place.

Several factors influence the level of serum inorganic phosphorus. These are: (1) vitamin D, through its action on increasing the absorption of calcium markedly and phosphate to a lesser degree, and by enhancing the renal excretion of phosphorus; (2) parathyroid hormone, which increases the urinary excretion of phosphorus and mobilizes it and calcium from bone; (3) growth hormone, which tends to raise the serum level of phosphorus; (4) functional state of the kidney, since phosphate retention occurs in severe renal disease; (5) the level of serum ionized calcium, since the concentration of calcium and phosphate ions tend to vary in a reciprocal manner.

Normal Concentration. The serum inorganic phosphorus content of normal adults varies between 3.0 and 4.5 mg. per 100 ml. Children, probably because of the effects of growth hormone, have a slightly higher concentration which extends from 4.5 to 6.5 mg. per 100 ml. The ingestion of carbohydrate temporarily lowers the concentration of serum phosphorus for a few hours. Because of the presence of phosphate esters in red cells, and of phosphatases in serum, it is essential that the serum be separated from the clot as soon as possible when performing serum inorganic phosphate determinations.

Increased Concentration. The concentration of serum inorganic

phosphorus is increased in individuals with *advanced renal insufficiency*, because of a decrease in phosphate clearance. The rise in the phosphate, however, is a late occurrence and this finding is associated with a poor prognosis (see creatinine section). Depending upon the degree of overdosage, and its duration, *vitamin D intoxication* is associated with either a normal or elevated serum inorganic phosphate concentration. Increased intestinal absorption of phosphorus and a mobilizing effect on the calcium and phosphorus of bone tend to raise serum values. Vomiting and diarrhea and the consequent dehydration may accentuate these effects. Both *true* and *pseudohypoparathyroidism* are characterized by an elevation in serum inorganic phosphate; in such cases values of 6.0 mg. per 100 ml. or higher are always found. Patients with *acromegaly* characteristically have serum phosphorus levels above 5.0 mg. per 100 ml. These values are associated with increased release of pituitary growth hormone. In children, on the other hand, growth hormone is normally responsible for a higher serum phosphorus than is found in adults. In dwarfism where there is deficiency of growth hormone, serum phosphorus may be below the minimum normal level for children of 4.5 mg. per 100 ml. Some patients with *osteoclastic bone metastases* may exhibit elevated serum phosphorus levels.

Decreased Concentration. Except for the fall in the concentration of inorganic phosphate that follows the ingestion of carbohydrate, decreased concentrations are seen in only three types of disease: (1) *Hyperparathyroidism:* The decrease in serum phosphate is more characteristic than the elevated calcium seen in this disease, since an elevated calcium may be obscured by a decreased protein concentration. (2) *Rickets*, *steatorrhea*, and *osteomalacia:* The low concentration of serum phosphorus in vitamin D deficiency is found more consistently than a low calcium. (3) Several *renal tubular abnormalities*, including the *Fanconi syndrome*. Apparently there is a defect in the renal tubular mechanism for reabsorption of many glomerular filtrate constituents, including phosphorus, which allows large amounts of the latter to be excreted in the urine. A lowering of serum inorganic phosphorus occurs in individuals receiving colloidal aluminum hydroxide for prolonged periods. Abnormally elevated levels may be entirely or partially restored to normal values in patients with renal insufficiency who receive the aluminum compound for this purpose.

Indication for Test. The indications and determinations listed for calcium apply in general to phosphorus. In addition to these, the test

is of value as an aid in diagnosing some renal tubular abnormalities or abnormal pituitary growth hormone activity in doubtful cases. In most instances, a simultaneous determination of serum calcium is desirable.

Method (Fiske and SubbaRow). A protein-free filtrate of serum is treated with aminonaphtholsulfonic acid in the presence of molybdate. The blue compound formed is proportional to the concentration of inorganic phosphorus present and can be measured colorimetrically.

Volume of Serum. 1 ml.

Calcium

Analytic data show that there are approximately 1100 gm. of calcium in the average 70-kg. adult. Although calcium exists in the body principally as a constituent of bone, it is present in small amounts in all tissue cells and extracellular fluids. In a manner similar to phosphorus, the calcium of bone and cells is in a constant state of exchange with the calcium of extracellular fluids. Calcium plays a role in regulating the degree of neuromuscular excitability, since there is an inverse relationship between calcium ion concentration and excitability. In addition, calcium is necessary for the blood clotting process and for the maintenance of normal membrane permeability.

Physiology (Albright and Reifenstein). Individuals are usually in a state of calcium balance, ingesting about 500 to 800 mg. daily in their food and excreting a like amount in the urine and feces. Some conditions, such as pregnancy and lactation, may cause an increase in the dietary requirement for calcium in order to maintain a normal balance.

Numerous factors affect the gastrointestinal absorption of calcium. For optimum absorption there must be: (1) a normal pH in the duodenum since acidity favors the formation of soluble calcium salts; (2) an adequate intake of vitamin D, a compound that facilitates calcium absorption; (3) a normal digestion of fat, since vitamin D is lost and insoluble calcium soaps are formed in steatorrhea; (4) the absence of an excess of phosphate, since it forms insoluble salts with calcium.

The presence of phytic acid (inositol hexaphosphoric acid), which is commonly found in some whole cereals, decreases calcium absorption since the two form an insoluble salt.

Calcium exists in at least two states in serum (McLean and Hastings). One form is combined with serum proteins and is probably physiologically inactive and incapable of diffusing across capillary membranes. Approximately 0.83 mg. of calcium per 100 ml. of serum is bound to each

gram of protein. The calcium not combined with protein is diffusible and consists principally of physiologically-active calcium ions and small amounts of an unionized, citrate-like calcium complex.

Numerous factors influence the concentration of total serum calcium. Since serum proteins combine with a definite amount of calcium, the protein concentration affects the total but not the ionized fraction of serum calcium. Hyperproteinemia is associated with an elevated calcium level while hypoproteinemia is accompanied by a decreased calcium concentration. The concentration of ionized calcium, however, is altered by the following: (1) Serum pH. Alkalosis tends to lower the calcium level and acidosis to increase it. (2) Intake of vitamin D. This vitamin elevates ionized calcium through its effect on calcium absorption and phosphorus excretion. (3) The hormone of the parathyroid glands. This is the most important factor controlling serum calcium levels and its effect is almost entirely limited to the ionized form. Two theories to account for the action of the hormone were originally advanced and both have now been substantiated. It appears that the hormone of the parathyroids has two actions, one on bone (the calcium mobilizing factor) and one on kidney (the phosphaturic factor). The calcium mobilizing action manifests itself by liberating calcium and phosphorus from bone, resulting in an elevated serum calcium concentration (Barnicot). In addition, the phosphaturic principle increases the renal excretion of phosphorus by decreasing its tubular reabsorption (Handler). This results in a lowering of the serum inorganic phosphorus level. The net effect of both actions is an elevation in the serum concentration of ionized calcium and a decrease in phosphorus. The urinary excretion of both ions is increased, the former because more ionized calcium is available for glomerular filtration.

The intake of calcium usually does not influence serum calcium levels. When the usual quantities of calcium are ingested, approximately 20 per cent is excreted in the urine. With a low intake, the percentage excreted by the kidney is slightly higher. The urinary excretion varies directly with the concentration of the ionized fraction in serum.

Normal Concentration. The concentration of serum calcium in normal individuals varies from 9.0 to 11.5 mg. per 100 ml. Approximately 4.5 to 5.5 mg. per 100 ml. are ionized, about 0.5 mg. per 100 ml. is present as a citrate-like complex, and the remainder is bound to serum protein. Since the concentration of calcium varies directly with the protein concentration, a calcium value can not be properly interpreted unless a simultaneous determination of serum protein is made.

Increased Concentration. An increased serum calcium concentration may result from either an increase in the bound calcium, due to a rise in protein concentration, or from an increase in the ionized fraction. Diseases characterized by increased serum proteins such as *multiple myeloma* and *sarcoid*, show an elevated calcium level, although inorganic phosphorus and alkaline phosphatase may not change. In both *hypervitaminosis D* and *hyperparathyroidism*,* an increase in ionized serum calcium and a hypercalcuria are seen. The differential diagnosis is usually made on the basis of clinical data and the determination of the concentration of serum phosphorus. This latter constituent is low in hyperparathyroidism while it is normal or high in vitamin D intoxication. In hyperparathyroidism, there is no increase in serum alkaline phosphatase activity if there is no bone involvement. If the phosphatase activity is increased, it can be used as a rough index of the degree of osteoporosis. If the diagnosis is not made early and corrective surgery instituted, renal insufficiency caused by precipitation of calcium salts either in the kidney parenchyma or as calculi may occur. Postoperative serial determinations of serum calcium in hyperparathyroidism will aid in the evaluation of parathyroid status.

In other metabolic diseases involving bone, such as *Paget's disease*, *Cushing's disease*, *Milkman's syndrome*, or *solitary bone cysts*, a differential diagnosis can be made by utilizing clinical data, x-ray findings, and chemical analyses. Differences in the serum concentration of calcium, phosphorus and alkaline phosphatase, and in the urinary excretion of calcium are of great value in this respect as indicated in Table 6.

Abnormal serum calcium values may be encountered in diseases other than those primarily involving bone. *Bed rest* and its attendant lack of movement will cause mobilization of calcium and phosphorus from bone but there will be no increase in alkaline phosphatase activity. *Patients ingesting large amounts of milk and alkali* for peptic ulcer may have a hypercalcemia without hypercalcuria and may show a normal or high serum inorganic phosphorus, with evidence of renal insufficiency.

Increased Concentration. In diseases characterized by a decrease in the ionized portion of the calcium, tetany may be seen. *Hypoparathyroidism*, either idiopathic or postoperative, is characterized by decreased calcium and elevated phosphorus levels in serum and by hypo-

* There are some cases in which it is necessary to perform calcium balance studies to establish the diagnosis of hyperparathyroidism since the blood findings may be inconclusive. With a low calcium diet, individuals with hyperparathyroidism will excrete abnormally large amounts of calcium in the urine.

TABLE 7.6

Diseases Characterized by Abnormalities in Calcium and Phosphorus Metabolism

Disease	Serum			Urine
	Calcium	Phosphorus	Alkaline phosphatase	Calcium
Vitamin D lack: rickets, vitamin D resistence, steatorrhea	→↓	↓	↑	↓
Hypoparathyroidism	↓	↑	↓→	↓
Milkman's syndrome	→↓	→↓	↑	↓
Idiopathic hypercalcuria	→↓	↓	↑	↑
Renal acidosis: tubular insufficiency or Fanconi syndrome	→↓	↓	↓	↑
Cushing's syndrome	→↓	→↓	↑	↑
Hyperparathyroidism	↑	↓	→↑	↑
Hypervitaminosis D	↑	→↑	→	↑
Multiple myeloma	↑	→↓	→	→↑
Paget's disease	→	→	↑	→↑
Metastatic carcinoma to bone	→↑	→↑	→↑	↑
Boeck's sarcoid	→↑	→	↑	↑
Solitary bone cyst	→	→	→	→
Polyostotic fibrous dysplasia	→	→	↑	↑
Bed rest	↑	→↑	→	↑
Milk and alkali	↑	→↑	→	↓

* ↑ = elevated concentration; → = normal concentration; ↓ = decreased concentration.

calcuria. *Pseudohypoparathyroidism* is a state in which there is an inability of the individual to respond to his own or to parenterally administered parathyroid hormone. The chemical findings are similar to those of true hypoparathyroidism.*

Tetany may also result from an *inadequate intake of vitamin D* or from *gastro-intestinal disease* when there is interference with the absorption of this vitamin. These conditions with tetany may exhibit a normal or low

* Two procedures have been suggested for making the differential diagnosis: (a) injecting parathyroid extract and measuring phosphate excretion. The characteristic increase in the rate of urinary phosphate excretion occurring in normals and hypoparathyroids, does not appear in the pseudohypoparathyroid; (b) injecting 100 to 200 units parathyroid extract daily for a week and following serum calcium and phosphorus values. The "true" hypoparathyroid patient exhibits an increase in serum calcium and a fall in phosphorus levels; these changes do not occur in the individual with pseudohypoparathyroidism. This latter test is considered more convincing than the former.

calcium but a low phosphorus and increased alkaline phosphatase activity. The tetany induced by the alkalosis of hysterical hyperventilation, however, will not show calcium, phosphorus or phosphatase abnormalities.

Low serum calcium values are seen in diseases associated with hypoproteinemia. Thus the *nephrotic syndrome*, some cases of *malnutrition*, *sprue*, and *cystic fibrosis of the pancreas* may have low total but normal ionized serum calcium levels.

Some degree of hypocalcemia may be seen with the phosphate retention that occurs with advanced *glomerulonephritis* or *nephrosclerosis*. In contrast to these types of renal disease are the findings of a normal or low serum calcium, low inorganic phosphorus and increased urinary calcium which may occur in the renal acidosis of tubular insufficiency or of the *Fanconi syndrome*. The fall in the serum calcium level that often accompanies *acute pancreatitis* is said to have poor prognostic significance.

Indication for Test. The concentration of serum calcium, together with total serum protein, inorganic phosphorus and alkaline phosphatase should be determined in individuals where there is clinical or x-ray evidence of bone disease. All patients with renal calculi should be investigated for the possibility of parathyroid hyperactivity. Serum calcium levels should be obtained on individuals with convulsions in order to differentiate hypoparathyroidism or severe vitamin D deficiency from epilepsy and hypoglycemic convulsions.

Serial determinations of serum calcium, phosphorus and alkaline phosphatase should be employed for guiding therapy in patients with hypoparathyroidism or vitamin D deficiencies.

Method. *Flame photometry.* Flame photometers are now available for the determination of serum calcium. Diluted serum is vaporized in the flame. Calcium molecules are excited and emit light which can be measured quantitatively at characteristic wave lengths.

Volume of Serum. 0.5 ml.

Chemical (Clark and Collip). Calcium in serum is precipitated as the oxalate, dissolved in acid and titrated with potassium permanganate.

Volume of Serum. 2 ml.

Sodium

The sodium ion, because of its predominant extracellular concentration (Table 7.4), is an important component of the osmotic factors involved in the distribution of water between extra- and intracellular tissue spaces.

In addition to its osmotic effect, the sodium ion plays a large role in the maintenance of normal acid-base equilibrium since it constitutes approximately 90 per cent of the total extracellular cations.

Physiology (Peters, 1952; Darrow and Pratt; Marriott, Edelman, Olney et al.). The body of the average 70-kg. adult contains approximately 80 gm. of sodium, of which 44 per cent is found in extracellular fluids and 35 per cent in bone. Infants, because of their relatively greater extracellular fluid volume, contain more sodium per kg. body weight than do adults. That all of the body sodium is not available for participation in physiologic reactions has been revealed by studies employing radioactive isotopes. The term "total exchangeable sodium" has been employed to describe the physiologically active portion of body sodium which approximates 82 per cent of the total. Thus, some 18 per cent of the total body sodium, mainly a portion of that contained in bone, is apparently inert. There are about 43 m.e. of exchangeable sodium per kg. body weight in adult males and 39 m.e. in females; the figure is larger in infants. As with body water these values vary inversely with body fat when expressed in terms of body weight. There is often no correlation between serum concentration and total exchangeable sodium.

Since sodium is found in both extra- and intracellular fluid, it is clear that cell membranes are not impermeable to sodium and that sodium ions inside and outside of cells are in a dynamic state of exchange. The concentration of sodium in extracellular fluids, however, is approximately ten times greater than its concentration in cell water (Table 7.4). The maintenance of this differential in sodium ion concentration between the fluids separated by the cell membrane is dependent upon active, energy-producing processes taking place within the cell.

Sodium is ingested in food in combination with chloride, phosphate, sulfate or other anions. Usually about three g. are ingested daily and gain access to serum probably by diffusion across the intestinal mucosa. Thus, about 4 per cent of the total body sodium is turned over daily. The sodium in serum is in equilibrium with that present in interstitial fluid, so that except for minor differences due to Donnan membrane equilibrium forces, the serum concentration is representative of all extracellular concentrations. The amount present in tissue cells cannot be measured at the present time without biopsy material.

The concentration and distribution of sodium in the body is governed by physical, chemical, endocrine and renal processes. As mentioned previously, a study of the movements of sodium requires mention of

shifts of water and sodium between the intra- and extracellular fluid. Since water and salt are not ingested in the same proportions as they are found in body fluids, one or both must be excreted if both the concentration and the total amounts of sodium and water in the body are to remain relatively constant. In general, this excretion takes place mainly through the kidney. With an increase in sodium intake, there is a temporary compensatory increase in extracellular fluid volume following the passage of water from cells into the interstitial spaces and some exchange of sodium for potassium in cells. Extracellular fluid volume increases temporarily both as a result of the retention of ingested water and the movement of water from the cells to the interstitial fluid. Both processes maintain the serum sodium concentration within narrow limits. In time, the excess sodium is removed by renal excretion. A deficit in the intake of sodium has the converse effect, so that there is an increase in the urinary excretion of water, a movement of sodium out of cells, and a decrease in extracellular fluid volume. In both types of conditions, the adjustments are such that concentrations of sodium, not absolute amounts present in the body, are maintained.

The pH of serum, through an effect upon sodium and potassium distribution, can alter the amount of sodium in the cell. A serum pH greater than 7.5 is associated with a movement of sodium and hydrogen ions into and a loss of potassium from cellular fluids. The reverse tends to occur with a lowering of the serum pH. The exchange of ions approximates the following relationship: $2Na^+ + H^+ \leftrightharpoons 3K^+$.

The steroids of the adrenal cortex are the hormones primarily concerned with sodium metabolism. Although the individual steroids of the adrenal have many effects on sodium, some producing an increase and some a decrease in the urinary excretion the predominant effect is to cause a decrease in the urinary output of sodium and to facilitate the renal excretion of water. In addition, these steroids aid in the maintenande of the normal intra-:extracellular ratio of sodium excess steroids increasing intracellular sodium and decreasing potassium. Lack of adrenal steroids is usually followed by an increased loss of sodium chloride in urine and may also be associated with a shift of sodium to some site, perhaps bone, where it becomes inaccessible to exchange. It has been shown that desoxycorticosterone and ACTH increase total exchangeable sodium and that lack of adrenal steroids may be accompanied by a decrease in this fraction of body sodium. Even though an individual may be in sodium balance, the total exchangeable body sodium can be increased or decreased in amount. Some of the gonadal steroids

cause a retention of salt and water; the edema or water retention seen premenstrually is explainable on this basis. The posterior pituitary and hypothalamus affect only the renal tubular mechanism for the handling of water and not the fate of sodium in the body.

Sodium is lost from the body almost entirely in the urine. The kidney, by adjusting its excretion of sodium and/or water, is the main factor in regulating the total body sodium and its concentration. It should be noted, however, that the stimulus for the excretion of sodium is not known; with an increased ingestion of sodium salts in a normal individual, the extra sodium will be excreted although no measurable change may have occurred in the serum concentration. Small amounts of sodium do appear in the feces and sweat under normal circumstances; concentrations in sweat and saliva are under the control of adrenal cortical steroids. Under abnormal conditions, the loss of electrolytes and water in sweat and by other exists, (e.g. gastro-intestinal fistula) may be considerable.

Normal Concentration. The concentration of sodium in normal serum is 138 to 145 m.e./L. Such values give no information as to the volume of distribution, the total amount present, nor of the amount present intracellularly. Clinical judgement concerning the state of hydration is important in evaluating the significance of a serum sodium level. Body water, especially extracellular water, affects the concentration of sodium by varying the volume available for distribution.

Increased Concentration. Hyperosmolarity, with increased concentrations of serum constituents, including sodium, is found in individuals with a *predominant body water deficiency*. Thus, patients with a relatively normal urinary output but whose water intake is inadequate because of coma, weakness, inability to swallow, or hypothalamic injury interfering with thirst mechanisms, may have a hypernatremia. Dehydration with raised serum sodium levels may occur if the fluid intake is insufficient for abnormal reasons; i.e. patients with uncontrolled diabetes insipidus, whose water intake is insufficient to balance urinary excretions, or those receiving a *high protein nasogastric* feeding when the fluid intake is not great enough for the kidneys to excrete an abnormally high solute load. *Diabetics in coma*, who are treated only with insulin and saline, and some patients with cerebral disease may also exhibit hyperosmolarity. The findings in patients with *diarrhea* are dependent on the relative losses of water and electrolytes; if proportionately more water than electrolyte is lost, a hypernatremia will be seen. The converse is seen where electrolyte loss exceeds that of water.

Decreased Concentration. Extracellular sodium levels are decreased when the relative loss of this ion from normal or abnormal routes of exit from the body exceed that of water. The findings in patients with *diarrheas*, *intestinal fistulas* and *Addisons's disease* are examples of this occurrence. With diarrhea, there is a loss of sodium, potassium and chloride from the body, the losses of sodium and chloride being approximately equal. With high intestinal fistulas, and the consequent loss of electrolyte and water, there is usually a greater loss of chloride than of sodium and potassium in the drainage. The resulting alkalosis is associated with an increased excretion of potassium in the urine and the passage of sodium into the cell. In Addison's disease, there is a decrease in extracellular and an increase in intracellular water attendant upon the increased urinary loss of sodium and chloride; segregation of sodium into some non-exchangeable compartment further depresses the serum level. At the same time, serum and presumably cellular potassium are increased because of retention. A deficit in body sodium accentuates a *metabolic acidosis*. The amount of sodium available for combination with bicarbonate is decreased by two factors: (a) a decrease in total body sodium and (b) an increase in the concentrations of phosphate, sulfate, and perhaps lactate and acetoacetate. In *renal disease with tubular dysfunction*, sodium, chloride and even potassium may be lost in excessive amounts in the urine. The loss may be ascribed to failure of base-saving mechanisms (exchange of H^+ or NH_4^+ for base). In other conditions, a defect of the renal mechanisms for handling salt ("salt losing nephritis") with symptoms and chemical findings indistinguishable from true adrenal cortical insufficiency, may result in a low serum sodium concentration.

In *uncontrolled diabetes mellitus*, sodium, potassium and chloride are lost in the urine, the first two ions in combination with the excess keto-acids. The "low salt syndrome" may be exhibited by some individuals on a salt restricted diet (rice diet or equivalent) where the intake of salt may be too small to balance the urinary excretion, or from prolonged therapy with ion exchange resins and attendant large gastro-intestinal losses. In some conditions, serum sodium concentration may be low, even though there is a marked increase in total body sodium. *Renal insufficiency, congestive heart failure, cirrhosis of the liver with ascites, the nephrotic syndrome and nutritional protein deficiency* are pathological states in which water retention may be excessive, leading to "dilution" of the increased quantities of extracellular sodium.

Indication for Test. Serum sodium together with potassium, chloride

and bicarbonate determinations should be performed whenever there are disorders leading to excessive loss of fluid and electrolyte from the body. As a rough index of the state of extracellular fluid volume, hematocrit values should also be determined. Serum sodium concentrations are desirable for guiding replacement of sodium deficits and are indicated for the diagnosis and in gauging the adequacy of therapy in Addison's disease. It is beyond the scope of this book to discuss replacement and maintenance therapy as regards fluids and electrolytes. For practical methods for the parenteral use of salt and water in the maintenance and replacement requirements of individuals in normal and abnormal water balance, see Hoffman, Weisberg.

Method. *Flame photometery:* Diluted serum is vaporized and the sodium ion is excited in a hot flame. The intensity of the resulting spectral line is proportional to the amount of sodium present and can be quantitated by means of a photoelectric system.

Volume of Serum. 0.1 ml.

Potassium

Potassium is the main intracellular cation; like sodium, which is primarily extracellular, it is an important component of the osmotic factors which determine the distribution of water between cells and extracellular fluid. In addition to this passive role, potassium actively influences cellular metabolism since it is a cofactor in many intracellular enzymatic reactions. Therefore, disturbances of the intracellular water and electrolyte environment lead to cellular and organ dysfunction. The concentration of potassium in extracellular fluids is approximately $\frac{1}{50}$ of that found in cell water and can be correlated with neuromuscular excitability and the conduction of electrical impulses of the heart.

Physiology (Darrow; Darrow and Pratt, Moore). There are approximately 130 gm. (3300 m.e.) of potassium in the average 70-kg. adult, of which more than 98 per cent is intracellular while less than 2 per cent is present in extracellular fluid. Ninety-five per cent of the total body potassium is exchangeable, as demonstrated by radioisotope techniques, although the rate of exchange of tissue potassium varies with the different organs. The potassium of bone, brain and red blood cells exchanges at a slower rate than the potassium of other organs. Since potassium is found predominantly intracellularly, the determination of total exchangeable potassium gives a measure of lean body mass; this latter parallels intracellular water. In adults, there are approximately 46 m.e. of exchangeable potassium per kg. body weight in males and 40 m.e. in

females. Infants have a relatively smaller potassium content per unit of body weight because their ratio of intracellular: extracellular water is much less than in adults.

Approximately 2 to 3 gm. of potassium combined as salts are ingested daily and exchanged with body potassium. Thus, approximately 2 per cent of the total body potassium in an adult is turned over daily; in an infant, where the intake of potassium is relatively greater, the daily exchange is 14 per cent.

Potassium is quickly removed from the extracellular fluids after it is ingested, as shown by isotope studies and by the slight, transient elevation in serum concentration. From serum, potassium is either transferred into interstitial fluid prior to entry into cells or across the renal glomerular capillary membrane into the glomerular filtrate. It is not known definitely whether potassium enters cells with glucose, phosphate or amino acids since all the evidence for the mode of entry is indirect.

Mudge has shown that the accumulation of potassium by rabbit kidney slices is an active process requiring aerobic oxidation and the generation of high energy phosphate bonds. Part of this energy is utilized to maintain the differential concentrations of sodium and potassium between intra- and extracellular fluids. However, it is not known whether the accumulation of potassium into the cell or the transfer of sodium out of it, is the primary process involved.

Many of the factors which control the distribution of sodium and chloride in the body also influence the movements of potassium, although the effect on the latter ion is usually not the same. The homeostasis of body potassium is maintained by chemical, physical and hormonal influences. Thus, intracellular potassium concentration varies with shifts of water which usually accompany alterations in extracellular sodium levels. Under the conditions of an increase in pH (alkalosis), associated with a decrease in chloride, there occurs an exchange of interstitial sodium for intracellular potassium; this exchange is not stoichiometric (vide infra). The deficit in cell potassium is mirrored in the serum findings; under these conditions, a hypopotassemia is the rule, since there is an enhanced urinary excretion of potassium chloride. With an inadequate caloric intake and the utilization of cellular protein for caloric purposes, potassium is liberated from cells and excreted, resulting in a hypotassemia.

Closely associated with the above influences are the effects of the adrenal cortical steroids which cause a retention of water and sodium and increase the urinary excretion of potassium which is derived from

cells. When excess steroids are present for a prolonged period, the deficit in intracellular potassium is reflected by a decreased potassium concentration in serum. At the same time there is an exchange of sodium ion for intracellular potassium. Furthermore, ACTH or desoxycorticosterone administration causes a decrease in total exchangeable potassium which may reflect the urinary loss. On the other hand, lack of adrenal steroids is accompanied by an increased concentration of potassium in cells and extracellular fluids and a decreased urinary excretion. Adrenal steroids can also increase salivary and sweat concentrations of potassium. In contrast to the steroids, glucose, insulin or epinephrine temporarily increases intracellular potassium at the expense of the extracellular ion, probably through the passage of a potassium-glucose-phosphate compound into cells.

Potassium is lost from the body mainly in the urine although small amounts are lost in the insensible perspiration.

The kidney handles potassium in a manner which differs from the handling of sodium in at least two ways. (a) There is evidence that potassium is excreted by the renal tubules so that its clearance may exceed that of the glomular filtration rate; no such mechanism has been described for sodium. (b) On a potassium-free diet, the urinary excretion of potassium is decreased only slightly from the excretion seen with an ordinary diet; on a sodium-free diet, the kidney (in a normal individual) conserves sodium.

Excessive amounts of potassium may leave the body under two conditions: (a) when intestinal secretions, with their relatively high potassium concentration, are lost through vomitus, diarrhea, or gastrointestinal fistulas, or (b) by increased urinary excretion in individuals with renal tubular abnormalities.

Normal Concentration. The concentration of potassium in the serum of normal persons is 4.0 to 5.0 m.e./L. In taking blood specimens, hemolysis must be avoided; otherwise potassium will be liberated from lysed erythrocytes. There is also a slow transfer of potassium from red cells to serum upon standing.

Increased Concentration. Hyperpotassemia, with a loss of intracellular potassium, is produced when increased cell membrane permeability occurs as a result of tissue damage. Under these conditions, serum potassium rises if the renal clearance of potassium cannot keep pace with the load presented. Thus, hyperpotassemia may be expected in *shock or circulatory failure* from any cause that reduces the oxygen supply to tissues. In *hypoventilation*, either central or peripheral in origin, there is often an increase in the concentration of serum potassium.

The loss of chloride and potassium that accompanies *diarrhea* or *intestinal fistulas* results in a deficit of potassium in the cell with replacement by sodium. Determinations of serum potassium may yield normal, high or low serum potassium levels, the concentration depending upon the relative loss of water, sodium and chloride and the degree of impairment of renal function attending these losses. With *uncontrolled diabetes*, the excessive breakdown of body cells and utilization of the protein for caloric purposes is accompanied by a loss of cellular potassium. The dehydration resulting from loss of water and base in the urine accentuates the increase in serum potassium concentration. *Adrenal cortical insufficiency* is another endorcinopathy which is associated with an elevation of serum potassium. The lack of adrenal steroids favors retention of potassium both in intra- and extracellular fluids. Some cases of *renal insufficiency* may show an elevation in the concentration of potassium although studies of total exchangeable potassium may reveal a deficit in the total amount of this cation.

Decreased Concentration. A deficit of body potassium, resulting from either a decreased intake or increased loss of this ion, *may* be reflected by a low serum level. With a *poor intake of food* or with *continuous intravenous glucose and saline therapy*, a hypopotassemia may occur because urinary excretion is maintained. A hypopotassemia, hypochloremia, an increased CO_2 capacity of serum and an elevated pH are found in individuals with vomiting, gastro-intestinal fistulas or after alkaline salts. The alkalosis seen with hyperventilation (and early in salicylate intoxication) is likewise accompanied by a hypopotassemia.

The cellular deficit of potassium that occurs during the development of diabetic acidosis and diarrhea is masked by dehydration, so that a high serum potassium may be seen. If potassium is not included in the therapy, a hypopotassemia may develop as fluids are replaced, resulting in muscular paralysis which may include the respiratory muscles. In these conditions, intracellular potassium is replaced by sodium. A hypopotassemia may be associated with some cases of *paralytic ileus* and *non-functioning intestinal anastomoses*. *Adrenal cortical hyperfunction* (including *primary aldosteronism*) produces a hypopotassemia, hypochloremia and alkalosis, with replacement of intracellular potassium by sodium. *Overdosage with cortisone* or *ACTH* produces the same results.

Periodic paralysis is a disease characterized by periodic muscular paralysis, with hypopotassemia during the attacks. In contrast to most causes of decreased serum potassium, where the potassium is lost in the urine or from other body secretions, in this disease the potassium moves

intracellularly. In some cases of *chronic renal disease*, potassium is lost in increased amounts in the urine, thus leading to a constant state of hypopotassemia and weakness or intermittent paralysis. In addition, individuals with an isolated functional derangement in the renal handling of potassium, causing hypopotassemia and hyperkaluria have been observed.

Indication for Test. Serum potassium determinations, in conjunction with those of other electrolytes, aid in evaluating the state of potassium in cells. Body potassium deficits may be indicated by a hypopotassemia and an alkalotic hypochloremia. It should be realized, however, that there is no simple way of estimating the state of body potassium as a whole. Many methods for this purpose have been proposed, including (a) estimation of the serum potassium level, (b) the performance of a potassium balance study, (c) the performance of a potassium loading test, (d) muscle biopsy, (e) the measurement of total exchangeable potassium, (f) the measurement of red blood cell potassium, and (g) electrocardiographic changes. All proposals suffer from some technical or practical disadvantage. Clinical evaluation of a patient is often as important as a serum level in judging the state of electrolyte balance or imbalance. It is advisable to estimate the serum potassium concentration in patients with unexplained, recurring paralytic attacks. Serial potassium levels, moreover, are of value in the diagnosis and treatment of adrenal cortical insufficiency and in preventing ACTH or cortisone overdosage.

Method. *Flame photometer:* The intensity of the characteristic emission line of potassium can be used to quantitate the amount present when a diluted sample of serum is vaporized in a hot flame.

Volume of Serum. 0.1 ml.

LIPIDS

The term lipid is used to designate a variety of naturally occurring esters, sterols and fatty acids that have the solubility properties of fats, i.e., low solubility in water and high solubility in "fat solvents" (ether, chloroform, petroleum ether, etc.).

The principal lipids found in human serum are cholesterol and its esters, phospholipids and triglycerides of fatty acids. (a) Cholesterol is a crystalline secondary alcohol of high molecular weight containing the steroid nucleus in its structure (Fig. 7.9). This nucleus is possessed by a group of physiologically active compounds, e.g., the vitamin D group, cardiac aglycones, bile acids, estrogens, androgens, adrenocortical

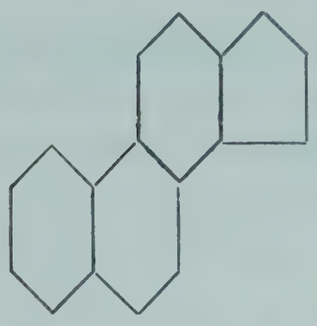


FIG. 7.9. Skeleton structure characteristic of sterols, steroids, bile acids, androgens and estrogens. (Courtesy of Best and Taylor: *The Physiological Basis of Medical Practice*, 6th Ed., The Williams & Wilkins Co., Baltimore, 1955.)

steroids and some carcinogens. Because of its hydroxyl group, cholesterol is able to form esters with fatty acids. (b) Phospholipids are fats containing a phosphate ester in the molecule, and in mammals, are composed of three types, namely, the lecithins, cephalins and sphingomyelins. The lecithins and cephalins are composed of glycerol esterified with two molecules of fatty acid and one of phosphoric acid linked to a nitrogenous base. In lecithin, the base is choline while in cephalin, the nitrogenous constituent is usually ethanolamine or sometimes the amino acid, serine. Sphingomyelin consists of a hydroxyamine (sphingosine) combined with a molecule of fatty acid and one of phosphoric acid attached to choline. (c) Triglycerides (neutral fat) are esters of glycerol and fatty acids.

The different lipids have some physiological features in common with respect to absorption, transport and synthesis which can be treated most adequately by discussing them together.

Absorption. The digestion of lipids takes place almost entirely in the intestines. Although the mechanisms of absorption are not clear, it is known that the presence of bile salts and pancreatic lipase are required for optimal absorption from the intestinal tract. Since triglycerides are insoluble in aqueous media, they can neither diffuse through intestinal membranes nor be acted upon by lipolytic enzymes until they are emulsified into droplets of colloidal dimensions. Bile salts in biliary secretions promote emulsification which is further enhanced by the formation of mono- and diglycerides from the partial hydrolysis of triglycerides. Under these conditions a stable fat suspension containing droplets less than 0.5 micron in diameter is formed. The major portion of the fat emulsion is partially hydrolyzed but a fraction passes directly through the intestinal cells to the lacteals and the lymphatic system (Frazer). Liberated long-chain fatty acids form water soluble complexes with bile acids that diffuse into intestinal cells. Here the fatty acids may be temporarily combined into phospholipid molecules before being recon-

verted to triglycerides in their passage to the lacteals. Triglycerides reach the lymphatic system in the form of chylomicrons, which are minute fat droplets of less than one micron in diameter enclosed by a film of protein (lipoprotein?) and enter the circulation through the thoracic duct. Glycerides of short-chain fatty acids pass through the intestinal wall and enter the portal blood system. Like triglycerides, the phospholipids undergo emulsification, hydrolysis and absorption, with a portion probably being absorbed unsplit. The fatty acids liberated from it are treated like those from triglycerides or cholesterol esters in the absorption process. The turnover rate of phospholipids in the intestinal mucosa is very rapid. Cholesterol is absorbed and transferred into the lymph system by a highly selective process, during which approximately 75 per cent of the sterol is esterified with fatty acids. Glycerol, choline and other portions of lipid molecules are either reutilized for synthesis in situ or are absorbed into the portal blood.

Under normal conditions, from 95 to 98 per cent of the fat contained in a meal is absorbed. Although feces contain about 5 per cent fat, all of this does not come from ingested food; about half is derived from intestinal secretions.

Transport. Despite the poor solubility of the lipid constituents in aqueous media, their concentration is high in limpid normal serum. The association of lipids with certain proteins to form giant colloidal lipid-protein complexes known as lipoproteins is responsible for their solubility in plasma. Lipids are transported primarily as lipoproteins; chylomicrons appear temporarily following fat absorption or in pathological states.

By chemical fractionation procedures, the lipoproteins have been separated from other serum proteins as two distinct sub-groups, the alpha-1 and the beta-1 globulins (Edsall). As shown in Table 7.7 the alpha lipoprotein molecules are of lower molecular weight, contain a lower lipid content and have a higher cholesterol: phospholipid (C/P) ratio than the beta species. The beta lipoproteins, comprising only 5 per cent of the total plasma proteins, nevertheless transport approximately 70 per cent of all the plasma lipids of fasting normal persons. In the majority of diseases in which a hyperlipemia is encountered, the beta lipoprotein fraction is elevated. Lipoproteins, when studied by means of free and paper electrophoresis, are identified by their characteristic mobilities as alpha-1 and beta-1 globulins. By use of the ultracentrifuge, however, many families of lipoproteins have been separated. Owing to its lipid content, lipoproteins are less dense than other serum

proteins. When subjected to high speed centrifugation after increasing the density of serum, a family of lipoprotein aggregates containing different proportions of protein, cholesterol, phospholipids and triglycerides float to the top of the centrifuge tube and form layers of varying density and refractive index (Gofman et al.). These lipoprotein groups separated at density of 1.063 are classified according to their flotation rates (S_f numbers) which vary from S_f2, the most dense to $S_f40{,}000$ (chylomicrons), the least dense. In general, the density varies inversely with the content of triglycerides (Table 7.7). The alpha lipoproteins are the most dense of all classes of lipoproteins and sink when centrifuged in serum of density 1.063; the beta group is next highest in density and corresponds to the S_f2–4 class (Table 7.7).

When fat is absorbed, chylomicrons pour into the bloodstream from the thoracic duct and produce a lactescence. This lactescence is rapidly cleared by a reaction involving heparin. Heparin interacts with a plasma protein and a tissue factor to form "clearing factor" which has the properties of a lipoprotein lipase. When acted upon by the "clearing factor," chylomicrons rapidly decrease their content of triglycerides and pass through the spectrum of low density lipoprotein classes, starting

TABLE 7.7

The Composition of Human Serum Lipoproteins of Various S_f Classes

Modified from Jones et al.

	Alpha Lipoprotein*	Flotation Units at Density 1.063			
		S_f4 Beta lipoprotein	S_f6–13	S_f13–40	S_f40–40,000
Total cholesterol	10%	35%	Decreasing steadily →		5%
Phospholipid	20%	30%	Decreasing steadily →		5%
Triglycerides	←Absent or very low→			Increasing steadily →	80%
Protein	65%	25%	Decreasing steadily →		5%
Approximate molecular weight	200,000	1,300,000	3,000,000	>3,500,000	
Density	1.07–1.12	1.040	1.035–1.015	1.015–0.96	<0.96

* Does not float at density 1.063.

with the least dense, and ending up in the beta- or alpha lipoproteins (Anfinsen). A visible lipemia found 12 or more hours after a meal is indicative of some abnormality in fat metabolism.

The concentration of the various lipids in plasma depends upon the net balance between the rates at which they enter the circulation and the rates at which they leave. The former are influenced by dietary intake, degree and velocity of absorption, rates of synthesis and release from liver, and mobilization from depots. The rates at which they leave the circulation depend upon factors of excretion, oxidation, storage and transformation into other compounds. Many of these processes are affected by endocrine factors. The liver is the organ most intimately involved in the temporary storage, synthesis, degradation, excretion, and transformation of the lipid constituents (*vide infra*). Fat mobilized from depots always goes to the liver as the first step toward its utilization.

Synthesis. In addition to coming from plant and animal products in the diet, the lipid components are also synthesized in the body from simple chemical compounds. Thus, glycerol arises from the reduction and hydrolysis of dihydroxy-acetone phosphate, a product formed during anerobic glycolysis. Saturated fatty acids are formed from the condensation of active acetate (acetyl coenzyme A) and/or acetoacetyl CoA. The "active" acetate for these reactions may arise from the catabolism of glucose, fatty acids or some amino acids. The synthesis of cholesterol is more complicated even though the same initial compounds are involved. Although many steps in the process are not known, there is good evidence that 3 molecules of acetyl CoA or 1 of acetyl CoA and 1 of acetoacetyl CoA condense to form isoprenoid units, that these latter combine to form squalene and that squalene is converted to the sterol, cholesterol (Bloch). All of the components of phospholipids except inorganic phosphate are also synthesized but the nitrogenous base, choline, may be a limiting factor in the case of the lecithins and sphingomyelins since choline is formed by the transfer of labile methyl groups from the amino acid, methionine, to ethanolamine; the supply of methionine is limited in diets low in protein or lacking in high quality proteins.

Cholesterol

Metabolism (Peters and Van Slyke, 1946; Katz and Stamler). Cholesterol is a constituent of protoplasm and is found in all body cells, mostly in the free form. It is derived from both exogenous and endogenous sources. The only dietary source of preformed cholesterol is that obtained by the ingestion of animal tissues or products.

Only negligible amounts of cholesterol are derived from ingested plant sterols since these compounds are poorly absorbed from the intestine, and that fraction which is absorbed is rapidly eliminated from the body. The addition of plant sterols or dihydrocholesterol to the diet inhibits the absorption of cholesterol, presumably by competing for absorption sites. A portion of the cholesterol in the gut escapes absorption and undergoes bacterial conversion to coprosterol, a saturated isomer of cholesterol. Coprosterol is excreted in the feces together with a small amount of insoluble dihydrocholesterol which is formed in the tissues and secreted into the intestine.

Although most tissues are able to synthesize cholesterol by the mechanism described previously, the liver is the main source of *plasma* cholesterol. Gould estimated that the total endogenous synthesis of cholesterol in man is 1.5 to 2 gm. per day but demonstrated that the ingestion of cholesterol greatly reduces its rate of synthesis by hepatic tissue. Cholesterol is secreted with bile, and once it enters the intestine, it is subjected to the same fate as ingested cholesterol. Cholesterol is also destroyed in the liver, primarily by conversion to cholic acid. The ring structure of cholesterol cannot be broken by tissue enzymes although the side chain may be oxidized and utilized for energy purposes. The three processes—absorption, synthesis and destruction—are closely coordinated since the total quantity of cholesterol in the body is relatively constant despite wide variations in dietary intake.

Both cholesterol and its esters circulate in plasma as components of of low density lipoprotein complexes (*vide supra*), the major portion appearing in the beta lipoproteins. Moderate changes in caloric content or composition of the diet have little effect upon serum cholesterol levels, although drastic, simultaneous curtailment of dietary cholesterol and fats such as occurs in starvation or with rice diets, results in a significant decrease. Differences in cholesterol concentration that are found among peoples in various parts of the world are ascribed to life span variations in the dietary and nutritional pattern rather than to racial, climatic or other factors. During pregnancy, cholesterol begins to rise around the third month and continues elevated until it recedes to normal a few weeks before term. Detectable amounts of cholesterol are not present in the urine of normal individuals but may appear during a massive proteinuria.

The role of cholesterol in health and disease is highly speculative. Its structural similarity to so many active, physiological compounds has encouraged many hypotheses concerning its role as a precursor. There is definite proof that cholesterol can be converted to pregnanediol and

some adrenocortical steroids but as yet no one has demonstrated that it is a precursor of estrogens or androgens. Cholesterol or giant lipoproteins containing cholesterol have been implicated as a causative factor in the development of atherosclerosis (see Katz and Stamler).

Normal Concentration. It is generally accepted that serum cholesterol values between 120 and 260 mg. per 100 ml. are normal for individuals below the age of 45 raised in the United States, but the upper limit may rise to approximately 300 mg. per 100 ml. for the 45- to 60-year age group. For age groups between 17 and 45, the average serum cholesterol concentration slowly increases at the rate of 2 mg. per 100 ml. per year and then slowly decreases after the age of 65 (Keyes *et al.*).

The serum cholesterol of the newborn is approximately $\frac{1}{2}$ of the young adult level but large variations are encountered. By 2 months of age, the concentration does not differ significantly from adult levels. From 68 to 76 per cent of the cholesterol circulates in the esterified form, regardless of age. Aside from slow variations with time, the serum cholesterol concentration of a healthy individual is rather constant and the ratio of cholesterol to phospholipids (C/P) is quite stable. The serum cholesterol levels of men and women are essentially the same but the C/P ratio may be lower in the latter group before the menopause because of the larger proportion of alpha to beta lipoproteins (*vide supra*).

Increased Concentration. Although hypercholesterolemia occurs in quite a few disorders (*vide infra*) its determination is of diagnostic aid in only a few conditions: *hepatic disease*, *hypothyroidism* and *xanthomatosis*.

Non-complicated, *extrahepatic obstruction of the common bile duct* is associated with a moderate or large elevation of total serum cholesterol concentration, depending upon the duration and etiology of the obstruction. The absolute amount of esters is normal or a little high but the percentage of esters is low because the rise is due to *free* cholesterol. The cholesterol usually returns to normal levels when the obstruction is relieved.

The finding of an elevated serum cholesterol was formerly used as an adjunct in the diagnosis of hypothyroidism, particularly in cases where the determination of the B.M.R. is not too reliable, e.g., in children and undersized or deformed individuals; this is being supplanted by measuring the serum protein-bound iodine or the uptake of I^{131} by the thyroid.

A hypercholesterolemia with a normal ester ratio is present in several types of xanthomatoses: primary essential xanthomatosis of the hypercholesteremic type and xanthomatosis with hyperlipemia secondary to other disease processes (Thannhauser).

In statistical studies with large groups, manifestations of deranged cholesterol metabolism are associated with coronary disease and atherosclerosis; these include (1) a moderately elevated serum cholesterol, (2) an increase in the C/P ratio and (3) an increase in the S_f 12–400 classes of lipoproteins. Because there is some overlap of individual values between the normal and patient groups, the question is still unsettled which of the above criteria will provide the best correlation with atherogenesis.

An elevated total serum cholesterol, for reasons not known, occurs in the following disorders but its determination has no particular diagnostic value: in the *nephrotic syndrome*, *uncontrolled diabetes mellitus*, and for several days following *acute hemorrhage*.

In spite of adequate insulin therapy and control of the disease, diabetics often exhibit a moderately increased cholesterol concentration. This may correlate with the incidence of vascular disease and other complications in this group of patients. Other hormonal factors influence cholesterol levels. Both total and esterified cholesterol in serum increase about 20% during vigorous treatment with cortisone. With extended ACTH administration, they first fall and then rise about 10% (Adlersberg et al.).

Decreased Concentration. *Hepatic disease with hepatocellular damage* is usually associated with a normal or low total serum cholesterol, with a decrease in the absolute amounts and percentage of the esterified portion. The drop in actual ester concentration helps to differentiate parenchymal damage from biliary obstruction. Serial determinations are of value for this purpose since the trend in obstruction is toward a rise in the serum levels of total and free cholesterol while total and esterified cholesterol concentrations tend to decrease with increasing hepatocellular damage. In the latter condition both esters and total cholesterol rise toward normal levels with recovery.

Total cholesterol in serum is usually low in *hyperthyroidism*, *anemias*, *acute infections*, *starvation*, diets low in fats and calories, and terminal states. For a time, the measurement of serum cholesterol was advocated as an aid in the diagnosis of hyperthyroidism but Peters and Man have reported a large series of cases in which there was no correlation between serum cholesterol concentration and thyroid activity.

Indication for Test. The determination of total, free and esterified cholesterol is of value in differentiating between obstructive and hepatogenous liver damage, in following the course of hepatocellular disease, and in diagnosing various lipemias. In hepatic or biliary tract disease, serial values are more informative than a single determination. With

only a minor degree of hepatocellular involvement, abnormal cholesterol values usually are not found. The thymol turbidity and cephalin-cholesterol flocculation tests are more sensitive indicators of liver damage.

Method. The Sperry and Webb revision of the Schoenheimer-Sperry method is recommended. Total cholesterol in an acetone-alcohol extract of serum is hydrolyzed with alkali and precipitated with digitonin. The precipitate is dissolved in acetic acid and color developed by the addition of acetic anhydride and sulfuric acid. The cholesterol concentration is proportional to the color intensity which is measured colorimetrically. Free cholesterol is measured in a similar fashion, omitting the hydrolysis. Cholesterol esters are obtained by difference.

Volume of Serum. 1 ml.

Phospholipids

Metabolism. The phospholipids, lecithin and cephalin, are structural materials required by all types of living cells. Sphingomyelin is found chiefly in brain and nerve tissue although it appears in small quantities in many other tissues, including plasma. Plasma phospholipids are composed of approximately 75 to 80 per cent lecithins, 15 to 20 per cent sphingomyelins and about 5 per cent cephalins (Hack). Significant amounts of each of the 3 types of phosphatides are likewise present in red cells. Radioactive phosphorus is incorporated very slowly into sphyngomyelin which demonstrates that this phosphatide is not very active metabolically.

Phospholipids are formed from both exogenous and endogenous sources (*vide supra*). The ingestion of fat stimulates phospholipid formation which takes place mainly in the small intestine and liver. In the fasting animal, however, the liver is the primary if not the only organ that can synthesize *plasma* phospholipids although other tissues can form cellular phospholipids (Chaikoff).

The role of the phospholipids in metabolism has not been clearly elucidated. It appears that the intestinal absorption of fatty acids may involve their temporary incorporation into phospholipids in the intestinal mucosal cells (*vide supra*). Although the phospholipids *per se* are not the vehicles for transporting fatty acids in plasma, their polar and non-polar groups enable them to form centers of attachment to the protein moiety of lipoproteins, thereby becoming an essential link between the other lipids and lipoproteins. This may explain why the plasma is not milky in appearance, despite the presence of a hyperlipemia, when the phospholipids constitute at least 30 per cent of the total lipids, e.g., in

xanthomatous bilary cirrhosis. Ingested lecithins, because of their choline content, have lipotropic activity, the ability to prevent the deposition of fat in or accelerate the removal of fat from the liver. Cephalins are involved in the blood clotting process for they constitute the lipid portion of thromboplastin.

Normal Concentration. Phospholipid concentration in serum may be expressed in terms of the entire compound or as lipid phosphorus. One mg. of lipid phosphorus represents approximately 25 mg. of phospholipids. The serum of normal persons contains from 150 to 350 mg. of phospholipids or 6 to 14 mg. of lipid phosphorus per 100 ml. As with cholesterol, the concentration of phospholipids is low at birth but reaches the adult level within a few months. Phospholipid levels vary with age in the same degree as does cholesterol. The serum concentration of phospholipids may be a little higher in women before the menopause than in men because of a higher concentration of alpha lipoprotein. The ingestion of fat produces a transitory rise in serum phosphatides.

Abnormal Concentration. *Xanthomatous biliary cirrhosis* is characterized by a marked increase (800–3000 mg. per 100 ml.) in the level of serum phospholipids. Some elevation in their concentration is found in the *nephrotic syndrome*, *uncontrolled diabetes mellitus*, *chronic hemorrhage*, *hypothyroidism* and in some of the *xanthomatoses* and *idiopathic hyperlipemias*. Serum phospholipids may be low in *hyperthyroidism* and in the anemias.

Indication for Test. The determination of phospholipids is of value in the differential diagnosis of xanthomatous biliary cirrhosis. It yields little information in other clinical conditions.

Method (Zilversmit and Davis). Serum phosphatides are precipitated with proteins directly from serum by means of trichloroacetic acid, the residue digested with perchloric acid and phosphorus determined as described for inorganic phosphorus. Phospholipids are calculated as 25 × lipid phosphorus.

Volume of Serum. 0.2 ml.

Triglycerides (Neutral Fat) and Total Fatty Acids

Metabolism. Triglycerides (esters of glycerol and fatty acids) constitute the third general type of lipid found in plasma and arise from exogenous and endogenous sources; their absorption, synthesis and transport have been discussed above. The fat stored in the depots, the chief fuel reserve of the body, is almost exclusively composed of triglycerides which are in a constant state of exchange with the triglycerides

in plasma and lymph. Triglycerides may be oxidized and utilized for energy, transformed to other compounds through the intermediate, "active" acetate, or stored in depots. Intestinal secretions contain triglycerides, part of which are excreted in feces.

In constrast to triglycerides which are a distinct entity, total fatty acids are derived from the laboratory hydrolysis of the serum phospholipids, triglycerides and cholesterol esters; the amount of free fatty acids in serum is negligible during the fasting state. Although total or esterified fatty acids must be determined analytically in order to estimate the triglyceride concentration, the latter constituent is the one of significance physiologically.

Normal Concentration. The concentration of triglycerides in serum varies from 0 to 9 m.e./L. or 0 to 250 mg. per 100 ml. while that of total or esterified fatty acids ranges from 9 to 17 m.e./L. or 250 to 500 mg. per 100 ml.

Increased Concentration. The serum concentration of triglycerides is usually elevated in *glycogen storage disease* (*Von Gierke's*) and in the disease states associated with the hyperlipemias mentioned in the cholesterol and phospholipid sections, with the exception of *essential xanthomatosis of the hypercholesteremic type* and *xanthomatous biliary cirrhosis.* Total fatty acid values are of necessity high in any serum containing increased levels of phospholipids, esterified cholesterol and triglycerides.

Indication for Test. The determination of triglycerides is necessary for the differential diagnosis of idiopathic hyperlipemias; it provides little information of clinical value in other conditions.

Method. *Triglycerides:* The serum concentration of triglycerides cannot be measured directly but is calculated from the results of 3 analyses (total or esterified fatty acids, phospholipids and esterified cholesterol) and is subject to their cumulative errors. The assumption is made that oleic acid is the acid present in all esters. Expressing all concentrations as mg. per 100 ml.:

$$\text{Triglycerides} = \frac{\text{Total fatty acids} - (0.67 \times \text{Phospholipids} + 0.73 \times \text{Cholesterol esters})}{0.95}$$

Method. *Total fatty acids* (Man and Gildea): Serum is extracted by refluxing with alcohol-ether and the extract is saponified with potassium hydroxide. The fatty acids are precipitated, separated and titrated with standard sodium hydroxide.

Volume of Serum. 2 ml.

The determination of esterified fatty acids (Stern and Shapiro) may be substituted for that of total fatty acids. The procedure is simple, more rapid and requires less serum.

ENZYMES

A large number of proteins with enzymatic activity circulate in plasma. These enzymes are normally elaborated by various glands or tissues and a portion of them appears in the blood. Alterations of enzyme activities in serum sometimes reflect a pathologic process occurring in the elaborating tissues or a disorder of the mechanisms for their excretion or disposal. Measurement of the serum enzymes mentioned below may be of aid in the differential diagnosis of certain clinical conditions.

Enzymes of serum appear in amounts too small to be separated quantitatively or identified in any way except by their action upon selected substrates. The concentration of an enzyme cannot be determined as mg. per 100 ml. or as m.e. per L. as is usually done with other serum constituents but must be measured under specific experimental conditions and expressed in terms of arbitrary units. The type and concentration of substrate, volume of serum, pH, time, temperature, electrolyte concentration, etc., affect the value of the unit. Hence, it is not surprising that various investigators employing different conditions of assay express their results in different activity units. It is not always possible to convert activity units obtained by one method into units measured in a different manner.

Alkaline Phosphatase

Physiology. There are several different enzymes in serum that are capable of splitting the terminal phosphate group from a variety of organic monophosphate esters. The one that exhibits optimum activity in alkaline solution (pH 9 to 10) is termed alkaline phosphatase; the one that has optimum activity in acid solution (pH 5 to 6) is acid phosphatase. It should be pointed out that the specific substrates upon which these enzymes act in the various tissues and the intracellular pH at the locus of action is not known. The arbitrary conditions of pH, substrate and electrolyte concentration employed in their measurement may be far removed from that which takes place in the body even though useful clinical information is obtained by these measurements. The two phosphatases have entirely different properties as shown by activation and inhibition studies.

Alkaline phosphatase has a wide distribution in the body and is pres-

ent in high concentration in bone, intestinal mucosa, and renal tubule cells, and in smaller concentration in liver and other tissues. The high concentration of phosphatase in osteoblasts has been suggested as providing a mechanism for supplying a high concentration of PO_4^{---} at the point in ossifying cartilage where it is needed for precipitation as a calcium salt. The role of alkaline phosphatase in intestinal mucosa and renal tubules, however, appears to be that of complementary dephosphorylation after the transport (absorption) of certain substances such as glucose across membranes. This is discussed in detail in a review by Moog.

The source of the alkaline phosphatase appearing in serum is not known, but under normal conditions, the bone-forming tissues appear to be the main contributor. A portion of the circulating enzyme is removed by the liver and excreted into bile.

Normal Activity. Most of the results in the literature are reported in terms of Bodansky units although this method is neither the best nor the simplest to carry out. For purposes of comparison with results obtained by other methods, the Bodansky unit is equivalent to approximately 2.5 King-Armstrong units and 0.8 phenol units. This relationship does not hold at all levels of activity and is merely an approximation.

The serum alkaline phosphatase activity of normal adults extends from 1.5 to 4 Bodansky or 1.3 to 3.5 phenol U. (Kaplan and Narahara). The activity of serum alkaline phosphatase is about 5 or 7 Bodansky U. at birth but quickly rises to a maximum of 10 to 20 U. during the first month where it remains for nearly 2 years; it slowly decreases with age until adult levels are reached in late childhood or adolescence. Values of 5 to 14 Bodansky U. or 4 to 12 phenol U. are considered normal for children between the ages of 2 and 14 years.

Increased Activity. Serum alkaline phosphatase is elevated in two types of pathologic conditions: (1) in diseases of bone in which there is increased activity of osteoblasts, and (2) in diseases of the liver and biliary passages in which the primary disorder is interference with the excretion of bile. In the former case, the rise in serum enzyme activity reflects increased production of enzyme with its escape into the blood stream. In the latter conditions the elevation is ascribed primarily to obstruction with regurgitation and consequent accumulation in plasma, but excessive hepatic formation may account for some of the rise.

In disorders of the skeletal system, the activity of serum alkaline phosphatase varies directly with the activity of cellular processes leading to the formation of new bone (Gutman, Tyson and Gutman). When

considered in conjunction with serum levels of calcium and phosphorus, the activity of alkaline phosphatase is of great value in the differentiation of various bone diseases and in following therapeutic measures. This is illustrated in Table 7.6 and has been discussed in the section on electrolytes.

In active *Paget's disease* (osteitis deformans), the activity of serum alkaline phosphatase is usually elevated and proportional to the amount of bone involvement. (Bodansky). In widespread involvement, enzymatic activity may range from 20 to 200 U.; in cases where the lesions are confined to a few bones, the activity is usually between 5 and 25 U. Children with untreated *rickets* always show elevated alkaline phosphatase activity that parallels the severity of the disease and which may lie between 20 to 200 U. The activity decreases with therapy and will finally return to normal when repair is complete.

In *hyperparathyroidsim*, an increase in serum alkaline phosphatase activity is seen only when there is mobilization of calcium and phosphorus from bone. Following parathyroidectomy, the enzyme activity returns to normal when bone recalcification is complete. *Osteoblastic tumors with metastases* involving the skeleton cause elevated phosphatase activity but this is not seen in osteolytic types of bone carcinoma if not accompanied by osteoblastic activity. The enzyme activity may be within normal limits or slightly elevated in *osteomyelitis*, *osteoporosis*, and *multiple myeloma*. It is usually within normal limits in calcinosis, chronic arthritis and tumors not involving bone. When interpreting alkaline phosphatase levels, it should be emphasized that the possible presence of liver disease must be ruled out and that the findings must be considered in connection with the concentrations of serum calcium and inorganic phosphorus (see electrolyte section).

The determination of serum alkaline phosphatase is of definite but limited value in differentiating between several types of disorders of the liver and biliary tracts, with or without jaundice. In the absence of excessive osteoblastic formation, an alkaline phosphatase activity greater than 10 U. indicates an *obstruction of the common bile duct* in about 80 per cent of cases, depending upon the type of obstruction. *Congenital biliary atresia* is the only type of extrahepatic obstruction with normal phosphatase activity. On the other hand, the *intrahepatic biliary* obstruction occurring in *hepatitis associated with cholangiolitis* and in *biliary cirrhosis* exhibit an elevated alkaline phosphatase activity. Activity below 10 U. occurs in about 85 per cent of cases of hepatogenous jaundice (Gutman and Hanger). Thus, about 15 per cent of both types of hepatic

disease overlap in the range of 4 to 10 units of alkaline phosphatase activity. By combining two different types of tests in these cases—alkaline phosphatase as a test for regurgitation jaundice of the obstructive type and cephalin flocculation as a test for hepatogenous damage—the differentiation between the two types of liver damage was sharpened considerably (Gutman and Hanger). The failure of serum alkaline phosphatase activity to rise in patients with severe hepatocellular disease exhibiting a high and rising serum bilirubin level has grave prognostic significance. This is attributed to a decreased hepatic production of the enzyme owing to severe necrosis and failure of liver regeneration. There is no increase in activity of serum alkaline phosphatase in hemolytic (retention) jaundice, but other tests are of more value in making the differential diagnosis. The above observations have no application to the differential diagnosis of jaundice in children because of the elevated but variable phosphatase activity normally encountered.

Decreased Activity. Decreased activity of serum alkaline phosphatase has little clinical significance. A lowered alkaline phosphatase activity is associated with *hypothyroidism* in children and sometimes is observed after surgical removal of the parathyroids. *Malnutrition*, *senility* and *anemia* may also be associated with lowered enzyme activity.

Indication for Test. The activity of serum alkaline phosphatase should be determined in patients with pathological processes involving bone or in whom biliary obstruction is suspected. Sometimes repeated analyses may be necessary to disclose an abnormality.

Method. The Kaplan and Narahara modification of Gomori's method is recommended because it is rapid, simple and accurate. Disodium phenylphosphate is hydrolyzed at pH 9.7 by serum alkaline phosphatase in the presence of Mg^{++} as an activator. The reaction is stopped by the addition of formaldehyde, and free phenol is measured colorimetrically after conversion to an azo dye. A unit is defined as one micromol of phenol liberated per hour by 1 ml. of serum.

Volume of Serum. 0.1 ml.

Acid Phosphatase

Physiology. Acid phosphatase is the enzyme present in serum that is capable of hydrolyzing monophosphate esters at pH 5. Acid phosphatase is found in liver, spleen, bone and kidney which are probably the sources of the relatively small amount of enzyme appearing in serum. The prostatic acinar epithelium of mature males, however, is a rich source of this enzyme and contains about 100 to 500 times as much acid

phosphatase per gram of tissue as that found in other organs. Semen also contains a high level of the enzyme which may be attributed to prostatic secretions. Under normal conditions, the acid phosphatase of the prostate does not enter the blood stream and this gland makes no contribution to the level of circulating enzyme. A portion of the serum enzyme is excreted in the urine. The role in bodily processes of acid phosphatase of serum, semen or the various tissues is not known.

Normal Activity. As with other enzymes, the value of the activity unit of serum acid phosphatase depends upon the method of measurement. In children and adults of both sexes, the upper limit of normal is 2.5 King-Armstrong U. or 1.5 phenol U. (Kaplan and Narahara).

Increased Activity. An elevation of serum acid phosphatase above 5 King-Armstrong U. or 1.5 phenol U. is indicative of *metastasizing carcinoma of the prostate.* The high same activity of acid phosphatase found in normal prostatic tissue appears in its metastasizing cells. As in the normal gland, the enzyme apparently does not enter the blood stream until spreading lesions penetrate blood and lymph channels. Once this occurs, the serum activity of acid phosphatase rises rapidly and remains high (Sullivan et al.). An enzyme activity between 3 and 5 King-Armstrong U. or between 1 and 1.5 phenol U. are suggestive of this malignancy and the test should be repeated at intervals. Approximately 15 per cent of patients with roentgenological evidence of metastasizing carcinoma of the prostate exhibit levels of serum phosphatase activity within the normal range while about 2 per cent of all types of patients without prostatic disease exceed the normal limits of enzyme activity; this latter happens most frequently in patients with hyperparathyroidism.

Decreased Activity. A low serum acid phosphatase activity has no clinical significance.

Indication for Test. Acid phosphatase activity of serum should be determined whenever metastasizing carcinoma of the prostate is suspected. Repeated determinations are helpful in following the progress of therapy of prostatic carcinoma since regression as a result of castration or treatment with estrogens is attended by a drop in serum acid phosphatase activity (Huggins and Hodges; Sullivan, et al.). Subsequent increases indicate progression of the disease through reactivation of old lesions or formation of new metastases. When the metastases invade bone, there may be a concomitant rise in serum *alkaline*-phosphatase as well, due to osteoblastic activity in the lesions.

There is no reason to perform the test on females.

Method (Kaplan and Narahara). The method is similar to that of alkaline phosphatase except that digestion is carried out at pH 5, Mg^{++} is not necessary for activation, and the reaction is stopped by the addition of sodium fluoride. Since erythrocytes contain a different acid phosphatase that will give high values if lysis occurs, it is essential to use serum that is not hemolyzed.

Volume of Serum. 0.2 ml.

Amylase

Physiology. Ingested starch is hydrolyzed to glucose in the digestive tract. The primary enzyme involved, amylase, initiates the splitting of starch and carries the process to the formation of dextrins and finally to maltose. Another enzyme, maltase, completes the hydrolysis to glucose. Amylase enters the digestive system in the juices secreted by the salivary glands, pancreas and perhaps by glands of the duodenal mucosa.

Although amylase is found in serum, the source of the enzyme under normal conditions is not known. It is possible to produce a temporary rise in serum amylase activity by experimental ligation of pancreatic or salivary ducts in animals but the activity soon returns to preoperative levels. The evidence indicates that a portion of the enzyme elaborated by pancreatic and salivary tissue cells can enter the blood stream through lymph channels when the acinar membranes are damaged. Amylase is excreted by the kidney and the enzyme activity of urine is about 2 to 6 times as high as that of serum. The level of urinary amylase is usually decreased when renal function is impaired.

Normal Activity. Amylase activity is usually measured by the Somogyi method and expressed in terms of his units. In normal individuals, serum amylase activity varies from 80 to 150 U. A value outside this range is not considered to be of clinical significance, however, unless it is less than 60 U. or greater than 200. Amylase is absent from the serum of newborn infants, appears at the age of 2 or 3 months and reaches the normal range around the age of one. The serum level of the enzyme is not further influenced by age, sex, nutritional state or dietary habits (Somogyi, 1941).

Increased Activity. The measurement of serum amylase activity shortly after the onset of symptoms of *acute pancreatitis* may be of great value in its diagnosis. This disease entity is very difficult to diagnose without the information contributed by the measurement of serum amylase or lipase activities. Amylase activity is consistently increased within 12 to 24 hours of an acute attack of pancreatitis and usually per-

or the preparation too thick, it may be difficult to distinguish neutrophils and eosinophils. In case of doubt call the cell a neutrophil, but better still move to a thinner portion of the slide and be guided by the size and shape of the granules rather than the color. A brighter light helps in differentiation.

Eosinophils usually constitute less than 3 to 4 per cent of the peripheral and bone marrow leukocytes. Sometimes in marrow preparations the eosinophils may be very large and irregular in shape (eosinophilic histiocytes).

Basophils (mast cells, basophilic granulocytes). Basophils are cells which have prominent dark blue staining granules which vary in size,

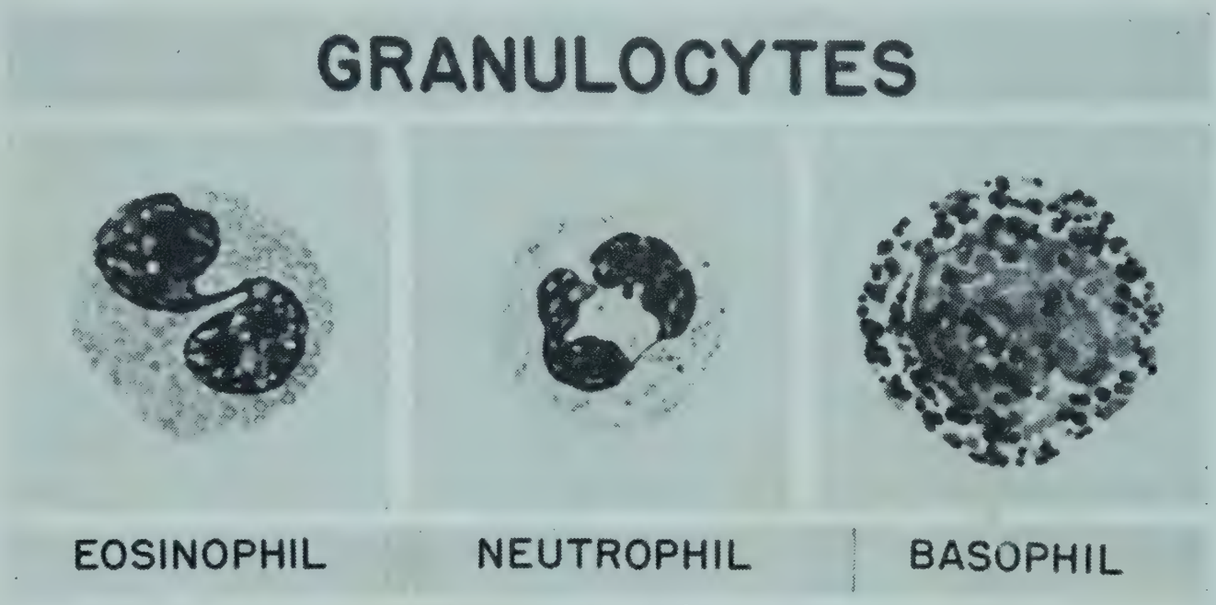


Fig. 2.2. Granulocytes

shape, and number, are unevenly distributed and overlie the nucleus (Fig. 2.2). Basophilic myelocytes, metamyelocytes, band and segmented forms are recognized, the criteria for differentiation being the degree of indentation of the nucleus and the size of the cell. Basophils, however, occur in such small numbers and have so little diagnostic importance that they are reported collectively as "basophils" rather than being subdivided into their developmental sequence.

There is no sharp line of differentiation between progranulocytes and basophilic myelocytes, for both have dark granules, a round nucleus, and early chromatin structure. The granules of a progranulocyte may be visible above or below the nucleus, but in a basophilic myelocyte the granules overlying the nucleus are conspicuous. In case of doubt between a progranulocyte and a basophilic myelocyte call the cell a progranulocyte.

Basophils which have few basophilic granules may be confused with

lymphocytes with azurophilic granules. The acidophilic granules of lymphocytes are usually lighter and spherical and are not visible over the nucleus. In case of doubt the odds are in favor of the cell being a lymphocyte.

The individual granules of basophils may stain unevenly and have ring or doughnut shapes which superficially resemble histoplasma capsulatum or protozoa.

Mature basophils are comparable in size to band or segmented neutrophils and constitute less than 1 per cent of the nucleated cells of bone marrow or peripheral blood.

Abnormal Granulocytes

Neutrophils with Hyperlobulated Nuclei ("P. A. poly."). In pernicious anemia and in other hyperplastic conditions in which there is a defect in maturation and delivery of cells, the neutrophils may be abnormally large and have more than five nuclear lobes.

Neutrophils with Toxic Granules. In severe infections and in other toxic states, the neutrophils may have granules which stain dark blue, although the nucleus is well differentiated.

Cells with Auer Bodies. Occasionally one sees in the early undifferentiated cells elongate structures in the cytoplasm. These structures are usually single but may be multiple. As a rule, they occur in cells in which no granules are obvious or in which there are few granules. The shape is usually bacilliform with needle, rhomboid, rectangular and occasionally corkscrew and granular variants. These granules are peroxidase positive. The finding of Auer bodies is presumptive evidence of leukemia and by most hematologists is considered to be evidence of myelocytic or monocytic types of leukemia.

Auer-like rods are seen in normal histiocytes. In the plasmocytes of some patients with plasma cell myeloma there may be conspicuous elongate structures which resemble Auer rods. Such structures are thought by Leitner to be a precipitated crystalline form of protein.

Lupus Erythematosus Cells ("L. E." cells). In smears of the buffy coat of heparinized, oxalated or defibrinated blood of patients with acute disseminated lupus erythematosus one may find within the neutrophils fairly homogeneous basophilic or pink staining material (Fig. 2.3). Cells containing such phagocytized material are known as "L. E." cells. It is thought that the phagocytized material is an abnormal protein which is engulfed by the neutrophils. Aggregations of the cells around particles of the same type are called "rosettes."

sists for 48 hours; levels of 2000 U. or more may be reached. In the majority of cases, the enzyme activity returns to normal within 2 to 6 days after the onset of the attack and does not rise again unless there is an exacerbation. The clinical usefulness of the test is confined to the first few days after the onset of symptoms. The fleeting rise in amylase activity in this disease is probably due to obstruction of ducts or inflammation, both of which damage acinar membranes.

Increased amylase activity has also been observed in *common duct obstruction by stones*, following *traumatic injury of the pancreas* and in some cases of *pancreatic malignancy*. A moderate rise may be found in *duct occlusion or suppuration of the salivary glands*, in *mumps*, acute alcoholism, and in individuals with *peptic ulcers that perforate near the pancreas* (Heifetz, Probstein and Gray). In chronic pancreatic lesions such as carcinoma, atrophy and chronic inflammation, serum amylase activity is usually within the normal range. When an elevation does occur, it is indicative of an acute exacerbation. A significant rise in serum amylase activity may be produced by the administration of opiates to patients, particularly those with biliary dyskinesia (Gross et al). This is caused presumably by a spasm of the duodenal-biliary musculature, closing the sphincter of Oddi at a time when the gland is secreting (Pfeffer et al.).

Renal impairment produces an elevated serum amylase activity because of decreased elimination of enzyme in the urine. Kidney damage must be ruled out when enzyme activity of serum is only moderately increased. The measurement of urinary amylase excretion is of no value as a kidney function test.

Decreased Activity. A decreased activity of serum amylase may be found in various types of *liver damage* (hepatis, cirrhosis, liver abscess, carcinoma, acute and chronic cholecystitis, etc.) but the status of the liver can be evaluated better by other hepatic function tests.

Indication for Test. The measurement of serum amylase activity should be made within 24 hours after symptoms suggestive of acute pancreatitis have appeared.

Method. The method of Somogyi (1938) is the one used most extensively and is preferred. Starch substrate is hydrolyzed by serum amylase in the presence of sufficient sodium chloride for maximum activation. Reducing substances are measured before and after the hydrolysis. The Somogyi unit is one mg. of reducing sugar liberated as glucose per 100 ml. of serum, under the conditions of the test.

Volume of Serum. 2 ml.

Lipase

Physiology. A group of enzymes termed esterase is able to hydrolyze various esters of alcohols and of fatty acids. Lipase, a member of this group, is capable of splitting triglycerides (neutral fat) into glycerol and fatty acids in addition to hydrolyzing certain simple esters of organic acids. The ability to split fats and oils is peculiar to lipase and is not shared to any extent by the other esterases of the body. This property has been utilized in some of the assay methods for lipase although it is possible to use certain simple esters as substrates under conditions of selective activation of lipase or selective inhibition of other esterases.

Lipase is elaborated by the acinar cells of the pancreas, the only gland containing lipase in high activity. It is secreted in pancreatic juice and is the most important fat-splitting enzyme found in the digestive tract. The small amount of lipase activity in gastric juice is physiologically insignificant. The presence of bile salts increases lipase activity in addition to acting as an emulsifying agent for fat.

A small degree of lipase activity is present in serum, and although its original source is not definitely known, it is presumed to come from the pancreas. When acinar membranes or duct canaliculi of the pancreas are damaged as the result of inflammation or trauma, serum lipase activity increases. Some lipase activity is found in the urine.

Normal Activity. Under normal conditions, the activity of serum lipase varies from 0.2 to 1.5 Cherry-Crandall U. or 0.8 to 1.5 Alper U. It is not perceptibly influenced by age, sex, diet or malnutrition.

Increased Activity. A sharp rise in serum lipase levels is observed very early in *acute pancreatitis*; this elevation may persist for 10 or 15 days (Comfort and Osterberg). Shortly after the onset of symptoms, serum lipase activity rises simultaneously with amylase activity but the former remains elevated much longer. In patients suspected of having acute pancreatitis, the amylase determination is the preferred test if a serum sample can be obtained within 36 hours after the onset of symptoms; its measurement is more accurate and less time consuming than that of lipase. The latter analysis, because of its longer period of elevation, is the one of choice when a greater time period has elapsed.

Serum lipase activity may also be elevated in cases of *carcinoma of the pancreas* because of the attendant obstruction or inflammation. Approximately 40 per cent of patients with pancreatic cancer have elevated serum lipase levels while the percentage is even higher if the malignancy involves the ampulla of Vater.

Serum lipase activity is also elevated in some other diseases involving

the pancreas. A high level has been observed in cases where *peptic ulcers perforate near the pancreas*, causing a secondary inflammation of this gland. When disease of the biliary tract extends to the pancreas, an elevated activity is usually present early after the onset of symptoms. When the pancreatic ducts are not occluded, the enzyme level is normal in malignancy of the biliary tract. An elevation of enzyme activity occurs in about 10 per cent of cases of cirrhosis and hepatitis and less frequently in chronic pancreatitis. As noted in the amylase section, the administration of an opiate drug may also cause an elevation in serum lipase activity in biliary dyskinesia.

Decreased Activity. A decreased level of serum lipase activity is of no significance.

Indication for Test. The measurement of serum lipase activity is of value as a diagnostic aid in cases of suspected pancreatic lesions.

Method (Alper modification of Goldstein et al.). Tributyrin is emulsified with Tween 20 and incubated in a buffered solution with serum. Liberated butyric acid is titrated in alcohol-ether solution with standard potassium hydroxide.

Volume of Serum. 2 ml.

MISCELLANEOUS

Sulfonamides

Physiology. Many derivatives of sulfanilamide (sulfapyridine, sulfathiazole, sulfamerazine, sulfaguanidine, etc.) have been prepared and tried therapeutically. At the present time, sulfadiazine, sulfisoxazole (Gantrisin), sulfamerazine, sulfasuxidine and sulfathalidine are among the most widely used sulfonamides.

The soluble sulfonamides are readily absorbed from the intestine, appear in the blood and other body fluids and are excreted by the kidneys. A portion of the circulating sulfonamide undergoes acetylation of the amino group attached to the benzene ring and becomes therapeutically inactive. This coupling appears to take place in the liver. Both the free and acetylated forms of the sulfonamides are excreted by the kidney. By contrast, sulfasuxidine and sulfathalidine are relatively insoluble and are employed for the treatment of intestinal rather than systemic infections.

As noted in the creatinine section, the deposition in renal tubules of sulfonamides or their acetylated products may cause widespread kidney damage. This may occur in the presence of an overdosage of the drug, insufficient fluid intake, or failure to administer alkaline salts. Recent

preparations (sulfisoxazole or triple mixtures) do not precipitate in the tubules as readily as the older ones.

Therapeutic Concentration. The optimum serum concentration of a sulfonamide varies with the specific drug used, but effective levels usually lie between 5 and 15 mg. per 100 ml. Sulfonamides are never found in the body unless the drug is administered.

Indication for Test. In patients receiving sulfonamides, serum levels should be determined semi-weekly to insure that therapeutic levels are achieved and to avoid the danger of overdosage.

Method (Bratton and Marshall). The amino group of sulfonamides in a protein-free filtrate of serum is diazotized and coupled with N-(1-naphthyl) ethylenediamine. The concentration of red azo dye is measured colorimetrically. The standard employed may be the same drug as measured, or sulfanilamide with appropriate conversion factors.

The acetylated drug must be hydrolyzed with acid prior to diazotization if this measurement is also desired. Since only the free form is therapeutically effective, there is no indication for this determination unless excessive acetylation is suspected.

Volume of Serum. Free sulfonamide—1 ml.

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CHAPTER 8

HEPATIC TESTS

By Clarence Cohn and Alex Kaplan

The liver is not only an organ with excretory activities but also one that participates directly or indirectly in numerous metabolic reactions occurring in the body. It is not surprising, therefore, that alterations in hepatic activities, as a result of disease may be reflected by abnormal concentrations of some plasma constituents and by aberrations in bilirubin metabolism. The qualitative or quantitative changes which occur in many components of blood and urine provide the basis for tests which serve to detect the presence of pathological processes affecting the liver; these types of laboratory procedures are commonly designated as chemical tests of liver function.

There are many inherent difficulties, however, in attempting to ascertain by laboratory methods whether a pathologic process has affected the activities of the liver (*vide infra*). For this reason it appears desirable to discuss in a general way the various laboratory determinations which have been utilized as hepatic tests and to indicate both their potentialities and limitations with regard to the information that can be derived from their use.

The tests employed for measuring the functional capacities of the liver ultimately depend upon the summation of many *integrated* enzymatic activities taking place within hepatic cells. As with all enzymatic reactions, those occurring in the liver are influenced by (a) the intracellular supply of substrates, oxygen, and energy, (b) the presence or absence of inhibitors, accelerators, co-factors, etc., (c) the composition of the intracellular milieu (pH, electrolytes, etc.), and (d) the activities of other organs in the body. Since many of these factors may be altered qualitatively or quantitatively without visible histologic changes in the liver, the correlation between the results of any given liver function test and demonstrable morphologic abnormalities may be fortuitous. By employing a group of carefully selected tests, the chances of properly evaluating the status of the liver are increased. Sometimes it is necessary to impose a load on the liver by administering a particular compound that is excreted, stored or conjugated before an aberration becomes evident.

Estimation of the presence or absence of hepatic dysfunction is complicated by the large functional reserve of the liver and its powers of re-

generation. Under certain circumstances as much as 85 or 90 per cent of the liver may be removed before impaired function is discernible by laboratory tests. Moreover, the various functions of the liver are altered unequally by hepato-cellular disease.

These factors often make it impossible, not only to quantitate the amount of hepatic tissue involved by a disease process, but also to differentiate diffuse damage from a localized dysfunction. A diffuse, minimal involvement of the liver may exhibit a more marked depression of hepatic function, as measured by laboratory tests, than a focal necrosis.

Despite the complexities of liver physiology and the limitations of liver function tests, the employment of selected procedures may yield information which is valuable clinically when pathology affecting the liver and biliary tract is present or suspected. Thus, hepatic tests may be used (a) to establish the presence (or absence) of disease, (b) to differentiate the types of jaundice and (c) to attempt to ascertain by the serial performance of these tests whether a disease process is progressing, regressing or remaining stationary. It should be emphasized that a disease affecting the liver can exist without being revealed by hepatic tests.

It is beyond the scope of this chapter to discuss the structure of the liver as related to its known physiology; Elias has described the present concept of liver architecture and its finer details and Wakim has summarized its physiology. Suffice it to say that two types of cells predominate in the liver parenchyma, hepatic parenchymal cells per se and reticulo-endothelial cells known as Kupfer cells. The cells are so arranged that the biliary system is separated from the vascular system by a layer of cells having a surface in contact with each channel. This arrangement allows for the transfer of bilirubin and other substances from blood to the biliary system and for an interchange of metabolic products, which may be in a state of dynamic equilibrium, between blood and hepatic cells. Little is known of the metabolic interrelationships of the hepatic parenchymal cells and the Kupfer cells.

A review of the literature on liver function tests reveals that innumerable laboratory procedures of varying degrees of complexity and reliability have been proposed to evaluate the functional status of the liver. In the discussion that follows the tests recommended are the ones that we have found most useful, reliable, simple and economical. Other tests, not mentioned, should not necessarily be considered inferior. For a further description of hepatic tests, the reviews of Hanger, Reinhold, Popper and Spellberg are recommended.

BILE PIGMENT METABOLISM

Useful information concerning the presence, course and often the type of disease associated with hepatic and posthepatic disorders can be obtained from:

(a) Measurement of serum bilirubin levels
(b) Detection of bilirubinuria
(c) Quantitation of urinary urobilinogen.

The normal and abnormal physiology responsible for alterations in concentrations and excretions of these pigments and the interpretation of the results of such measurements are therefore considered in the following sections.

Serum Bilirubin

Bilirubin is one of the end-products of hemoglobin breakdown in the body and is excreted solely by the liver via the bile. Elevated concentrations in serum are of clinical importance in the diagnosis and evaluation of liver damage but pathologic conditions other than liver disease can raise the bilirubin level in the blood stream.

Hemoglobin is a conjugated protein, composed of an iron-pyrrole complex (ferroprotoporphyrin) linked to a protein, globin. The ferroprotoporphyrin consists of four heme molecules, each composed of protoporphyrin and iron, associated in a complex. When erythrocytes are destroyed in the hepatic and extrahepatic portions of the reticuloendothelial system, hemoglobin is released and degraded in these cells. The protein portion enters the nitrogen "pool" of the body and is utilized in catabolic or anabolic pathways; the iron is returned to the iron pool and is reutilized for hemoglobin synthesis. By contrast, the porphyrin fraction of the hemoglobin is not utilized again but is degraded and excreted; one scheme for this process is shown in Figure 8.1.

As illustrated in Figure 8.1, the first step in Watson's theory of the decomposition of hemoglobin is the opening of the porphyrin ring, leading to the formation of choleglobin, a green colored product. Choleglobin loses iron and biliverdinglobin is formed, which is then reduced to bilirubinglobin. This latter product is released into the blood stream and lymph and carried to the liver where the globin is detached prior to the excretion of cholebilirubin into bile. After reaching the gastrointestinal tract, cholebilirubin is reduced by bacterial action to other pigments, collectively called urobilinogen, about 50 per cent of which is absorbed, circulated and finally excreted by the liver. During its circulation, a minimal amount enters the renal glomerular filtrate and is excreted in the urine.

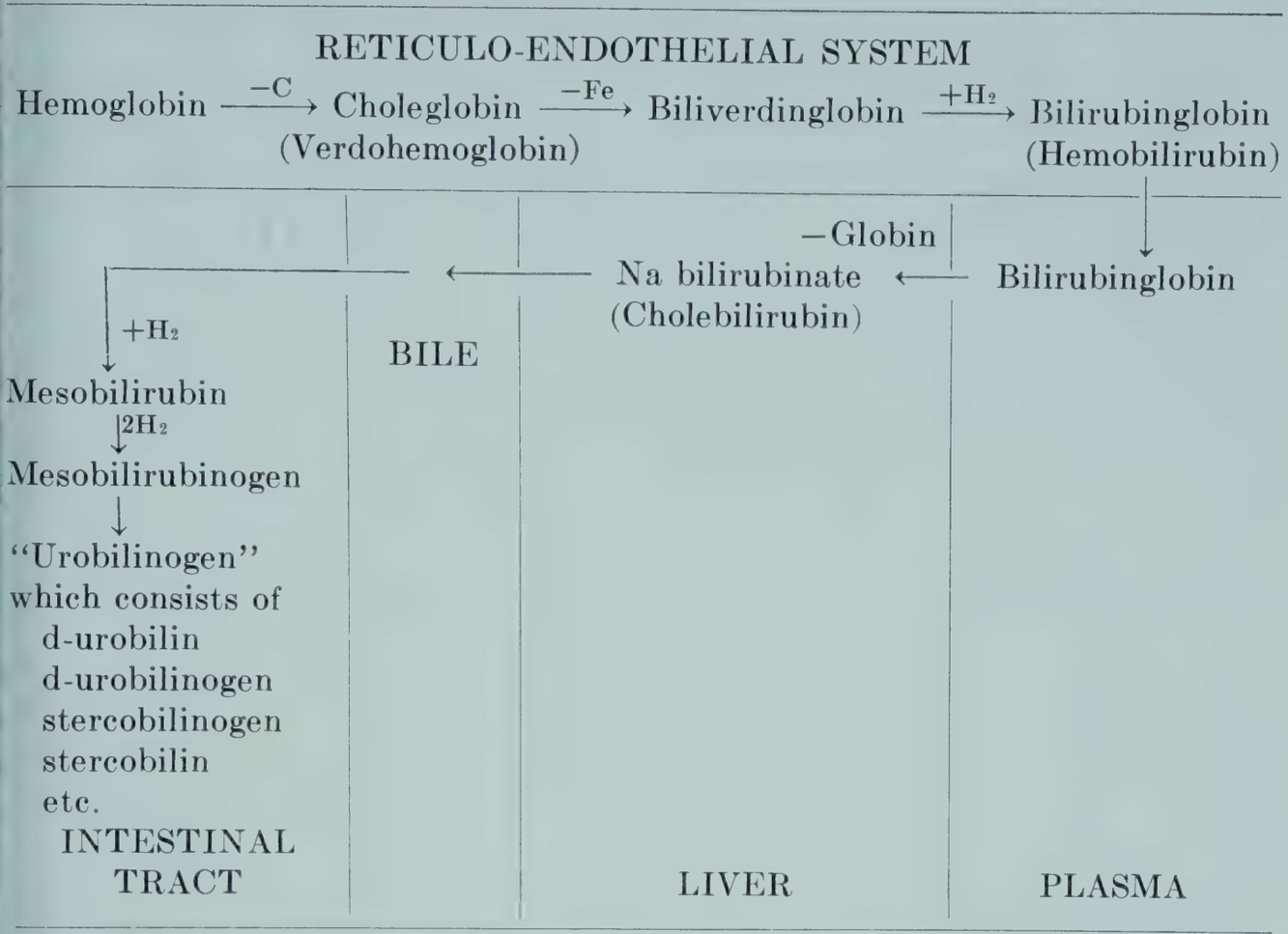


FIG. 8.1. Schematic diagram illustrating the breakdown of hemoglobin to bile pigments (Watson).

Since bilirubin is derived from hemoglobin, the amount formed daily is a function of the total circulating red cell mass, its hemoglobin content and the turn-over rate. The normal life span of the red blood cell is approximately 120 days; therefore, 0.83 per cent of the circulating red cells are destroyed daily. Each gram of hemoglobin that is degraded yields 35 mg. of bilirubin and urobilinogen; hence a 70-kg. individual with 750 gm. of circulating hemoglobin breaks down 6.25 gm. of the pigment and forms 220 mg. of bilirubin daily. The average time that bilirubin circulates in serum before excretion by the liver is 1.5 hours, indicating a slow clearance of this compound by the liver.

From the results of bilirubin clearance studies, it is apparent that the liver can clear exogenous bilirubin administered by intravenous injection at a rate that is greater than the normal endogenous load of bilirubin. Under such conditions of loading the liver clears bilirubin at a rate which is approximately proportional to the square of the concentration in plasma.

There is increasing evidence that bilirubin exists in serum in more than one form. Much of the controversy of the past over the state of serum bilirubin arose from interpretation of data concerning the rate of

reaction of bilirubin with Ehrlich's diazo reagent. It was believed that bilirubin existed in serum in two forms: (a) one form that was presumed to circulate free of protein, reacted promptly with the aqueous diazo reagent (direct or 1 minute bilirubin); (b) the second type was postulated as being attached to protein, requiring the presence of alcohol before it would react completely with the diazo reagent (indirect bilirubin). The indirect bilirubin was obtained by subtracting the 1 minute value from the total bilirubin. It was further hypothesized that the indirect or protein-bound bilirubin was the product of the reticulo-endothelial system and the pigment was detached from its protein by passage through the liver.

These assumptions have been challenged by a number of workers. Thus, the formation of 1 minute bilirubin by the hepatectomized animal (Ingle) cannot be reconciled with the above scheme. Furthermore, the separation by crystallization or chromatography techniques (Najjar and Childs; Cole and Lathe) of serum bilirubin into 2 or more fractions that were free of protein has demonstrated that diazo-reacting pigments of serum exist in several forms with different physico-chemical properties.

It has been shown that there are at least three pigments in serum, including two fast polar ones, which are soluble in water, give a direct diazo reaction, and are found to predominate in the serum of individuals with biliary obstruction. The nature of these compounds is unknown but they are closely related to true bilirubin. A slow-moving, less polar component, which moves similarly to true bilirubin, is soluble in organic solvents, requires alcohol for the diazo reaction, is found in the serum of individuals with hemolytic anemia and which is considered to be true bilirubin has been demonstrated. Since both reactions (direct and indirect) occurred in the absence of protein, it is obvious that alcohol could not participate in the reaction by splitting bilirubin from protein. These results shed no light, however, on the mechanisms controlling the formation of the 2 types of diazo-reacting pigments or the role of the liver in this process.

From the standpoint of diagnosis, not all investigators have obtained more information from the 1-minute serum bilirubin level than from the total in patients with hepatic and/or posthepatic lesions. In general, both the direct and indirect types of bilirubin tend to rise more-or-less proportionately as the jaundice increases. The use of a qualitative test for the presence of bilirubin in urine, increases the probability of detecting an individual with liver or biliary-tract disease and makes un-

necessary the measurement of the 1-minute bilirubin. Although the bilirubin liberated during hemolytic processes characteristically does show a delay in color development with the diazo reagent and does not appear in the urine, better tests than those for 1- and 30-minute bilirubin exist for demonstrating hemolytic diseases.

From our experience, we recommend performing the measurement of total bilirubin only and omitting the 1-minute bilirubin test.

The concentration of bilirubin in normal serum varies between 0.1 and 1.0 mg. per 100 ml. This concentration represents a balance between the average 250 mg. liberated into plasma daily by the reticulo-endothelial system and its rate of removal by the liver and excretion in bile. Rather marked daily variations in serum bilirubin levels, up to 0.7 mg. per 100 ml., have been observed in normal individuals.

Levels above 1.4 mg. per 100 ml. are abnormal and, depending upon the concentration and unknown factors, will be associated with clinically detectable or "latent" (clinically invisible) jaundice. Usually values above 2.5 mg. per 100 ml. are required before jaundice becomes clinically evident in adults. This is attributable to the affinity of elastic tissue for bilirubin, thus imparting a characteristic yellow color as the concentration exceeds a critical level. In children, whose elastic tissues are poorly developed, serum bilirubin values must reach higher values before jaundice is detectable.

Since the liver normally can handle much more bilirubin than it usually does, the jaundice associated with hemolytic syndromes results from the fact that the maximal excretory transfer capacity of the liver for bilirubin is exceeded although the rate of bilirubin clearance may be normal. Some concomitant hepatic impairment, resulting from anoxia and products of red cell degradation, may, however, on occasion depress the clearance of bilirubin.

Several bases for the classification of jaundice exist; none is completely satisfactory because considerable overlapping of the various categories occurs. In addition, few disease processes, acting on the liver for any length of time, remain a "pure" type. Thus, with chronicity, a post-hepatic obstruction gradually produces hepatic cellular dysfunction. Anoxia resulting from congestive heart failure (prehepatic jaundice) may lead to functional and then to histological changes in the liver (hepatic jaundice).

A system of classification based upon anatomic, physiologic, therapeutic and etiologic considerations appears below.

Classification of Jaundice

(Modified from Ducci; Young; Rich; Dameshek)

Anatomic	*Physiologic*		*Therapeutic*
Prehepatic	Excessive blood destruction	Hemolytic	Non-obstructive (medical)
Hepatic	Functionally subnormal liver cells; Necrosis of liver cells	Hepatocellular	Non-obstructive (medical)
Posthepatic	Biliary obstruction		Obstructive (surgical)

1. *Prehepatic jaundice:* This is caused either by overproduction of bilirubin or by anoxia interfering with normal hepatic function
   a. Hemolytic anemia. The increased hemolysis may be attributed to:
      (1) Due to "intrinsic" defects of the red cell (Short red cell life span of patient's cells in normal circulation):
      Hereditary spherocytosis
      Hereditary leptocytosis (Mediterranean anemia-thalassemia)
      Hereditary sickle-cell anemia
      Other hereditary hemoglobinopathies (C, D, E, and combinations with thalassemia or sickle-cell disease)
      Other hereditary "abnormalities":
        Familial non-spherocytosis
        Hereditary hypochromic anemia with high serum iron
        Others, not well classified, including primaquine hypersensitivity
      Paroxysmal nocturnal hemoglobinuria
      Pernicious anemia
      Paroxysmal march hemoglobinuria
      (2) Due to "extrinsic" mechanisms (Red cell life span altered by an outside factor):
      Bacterial, viral (Bartonella, *B. welchii*, etc.)
      Parasites (malaria)
      Chemical
      Auto-immune:
        with agglutinins—hemolytic anemias
        with hemolysins—hemoglobinurias
      Iso-immune:
        Transfusion reactions
        Rh disease
        Others
      Hypersplenic
      "Symptomatic"
        Disseminated lupus
        Periarteritis nodosa
        Lymphosarcoma
        Others

b. Decreased hepatic blood flow:
   (1) Congestive heart failure. If anoxia is great enough, jaundice will occur.
   (2) Shock syndrome.

2. *Hepatic jaundice:** Functionally subnormal, abnormal and necrotic hepatic cells are incapable of transferring bilirubin into the bile passages at a normal rate. The following causes may be implicated:
   a. Congenital abnormality:
      (1) Familial nonhemolytic jaundice. This is a disease characterized by an inherited, diminished ability to remove hemobilirubin, although all other liver function tests are normal.
   b. Chemical: Chloroform, carbon tetrachloride, phosphorus, etc.
   c. Infectious agents:
      (1) Viral—Infectious hepatitis, homologous serum hepatitis, yellow fever, infectious mononucleosis (?)
      (2) Spirochetal—Weil's disease, syphilis, relapsing fever
      (3) Bacterial—Pyogenic liver abscess, tuberculosis of liver, brucellosis
      (4) Protozoal—Amebic hepatitis and abscess
      (5) Metazoal—Schistosomiasis, hepatic distomiasis (liver flukes), hydatid cysts
   d. Neoplastic: Cystadenoma, hemangioma, primary or metastatic carcinoma, metastatic sarcoma
   e. Nutritional: Fatty liver, hemosiderosis, kwashiorkor, portal cirrhosis
   f. Metabolic: Hyperthyroidism, hemachromatosis, Gaucher's disease, Niemann-Pick disease, amyloid disease, glycogen storage disease.
   g. Anoxial: A deficient oxygen supply to hepatic cells, irrespective of cause (decreased blood flow, severe anemias).

3. *Posthepatic jaundice:*† Obstruction of the biliary passages, either intra- or extra hepatic, prevent otherwise normal cells from transferring bilirubin into the bile canaliculi. It has also been suggested that there is spilling over of bilirubin from the bile canaliculi into the blood stream when obstruction exists. The obstruction may be caused by:
   a. Congenital abnormalities:
      (1) Atresia of bile ducts
      (2) Congenital familial nonhemolytic jaundice with kernicterus; this disease is characterized by a genetically determined characteristic for a diminished ability to clear bilirubin, both endogenous and exogenous, although all other liver function tests are normal. Pathologically, kernicterus and bile thrombi in the biliary canaliculi with some periportal fibrosis, are found.
      (3) Inspissated bile in biliary passages

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* Hepatic jaundice refers only to lesions or dysfunction of the parenchymal cells per se.

† Posthepatic jaundice refers to disease processes of the biliary passages, either intra- or extrahepatic. Intrahepatic disease affecting the bile canaliculi has been classified as a hepatocanalicular lesion and included under the heading of hepatic jaundice by Ducci. This shows how all classifications are somewhat arbitrary.

(4) Fibrocystic disease of the pancreas may be associated with jaundice if the intrahepatic biliary ducts are obstructed with mucus.

b. Calculi: Calculi in the common bile duct originating in the liver or gall bladder or pancreatic calculi blocking the ampulla of Vater.

c. Inflammation: Cholangitis or cholecystitis with ascending cholangiolitis producing obstruction, in addition to increased permeability with leakage; occasional cases of acute or chronic pancreatitis with compression of common bile duct; scars around duct, biliary cirrhosis.

d. Carcinoma: Of common bile duct, of ampulla of Vater, of head of pancreas; malignancies, either primary or metastatic of lymph nodes around common bile duct.

There are several aspects of bilirubin metabolism that are not understood at the present time which deserve mention. It is not known why, with complete biliary obstruction, the serum bilirubin level does not continue to rise but tends to level off at about 50 mg. per 100 ml. Likewise, there is no explanation why the serum bilirubin concentration does not become elevated in some patients with partial biliary obstruction or when extensive loss of hepatic tissue occurs.

The concentration of serum bilirubin should be determined in patients with suspected liver disease whether or not clinical jaundice is evident. If other function tests indicate the presence of hepatic damage, serial determinations of bilirubin should be carried out even though the first bilirubin value is within the normal range. Such a procedure aids in ascertaining whether the patient is in the pre- or post-icteric stage of hepatitis, or whether hepatitis without jaundice is present. In the presence of clinical jaundice, serial determinations of bilirubin, in conjunction with other pertinent tests, aid in the evaluation of the disease process. Serum bilirubin levels are of no value in differentiating between acute hepatitis and obstructive conditions since a steady increase in concentration may occur in both conditions. Large fluctuations in serum bilirubin levels of jaundiced patients may indicate either a ball-valve type of obstruction or one associated with processes compressing the common bile duct and subject to exacerbations and remissions. As discussed above, the performance of the 1-minute bilirubin test is of limited value. Weekly or semi-weekly serum bilirubin determinations should be performed during convalescence from a hepatitis or after relief of an obstruction as an aid in determining the progress of recovery or the success of the operative procedure.

It is now quite evident that attacks of hepatitis can occur in the absence of hyperbilirubinemia. Both infectious hepatitis and infectious mononucleosis may occur without overt jaundice.

The method of choice for the determination of bilirubin is the one described by Malloy and Evelyn.

Bilirubinuria

Bilirubin can not be detected by the usual techniques in urine from normal individuals. Positive tests for bilirubinuria are therefore prima facie evidence for disease of the liver or the extrahepatic biliary system. In the early stages of acute hepatitis, bilirubin often is found in the urine before serum concentrations have risen above the normal range; this test is exceedingly valuable in studying individuals in whom hepatitis is suspected before jaundice is clinically discernible or in those who develop hepatitis without jaundice. By contrast, later in the disease, the tests for urine bilirubin may become negative at the time that hyperbilirubinemia is still present.

The mechanisms responsible for the appearance of bilirubin in urine are unknown. It has been suggested that bilirubin appears in the urine when the serum level of the 1-minute (filterable?) bilirubin increases. However, an examination of the data on which this theory is based reveals that there is no good correlation between 1-minute serum bilirubin levels and the appearance of bilirubin in urine. Further evidence that discredits this explanation is the aforementioned fact that bilirubin may disappear from urine during recovery from acute hepatitis although the serum level of the 1-minute pigment is still elevated.

Hemolytic diseases, with or without jaundice, are not accompanied by the excretion of bilirubin in urine ("acholuric jaundice"). The results of tests for urine bilirubin therefore enable one to differentiate hemolytic jaundice from other types. Once the presence of hepatic disease has been established, little information can be gained by the continued measurement of urine bilirubin, especially if serum bilirubin levels are being followed. The Harrison-Fouchet Spot test or the "Bilazo" (Free and Free) test are recommended for detecting urinary bilirubin.

Quantitative Urinary Urobilinogen Excretion

As noted in the section on bilirubin, the final measurable degradation products of the porphyrin ring of hemoglobin have been designated "urobilinogen." These products, (tetrapyrroles) of which at least six have been characterized, are formed by the action of gastrointestinal bacteria on the bilirubin excreted into the gut via the bile. Urobilinogen reacts with Ehrlich's aldehyde reagent to give a red color. Further breakdown of these tetrapyrroles in the gut to mono- and dipyrroles may

occur but these do not react with the aldehyde reagent. The measurement of fecal urobilinogen usually approximates the amount expected on the basis of a calculated red cell breakdown. However, this agreement may be fortuitous in that two possible reactions which influence the results in opposite directions may occur and cancel each other out. Thus, there is evidence for the production of some 10 to 20 per cent more bile pigment than can be accounted for by the red cell destruction; this could be counter-balanced by the conversion of tetrapyrroles to mono- and dipyrroles. Normal individuals excrete in their feces 50 to 250 mg. of urobilinogen daily.

Approximately 50 per cent of the urobilinogen formed in the intestines is absorbed in the colon, circulated in blood and transferred by the liver into the bile. Some of the pigments enter the renal glomerulus during their circulation through the kidney and are excreted in the urine. In a normal individual, the urinary excretion varies between 0 and 2 mg. in 24 hours.

With hepatic disease or with obstruction of the biliary passages, alterations in the excretion of urinary urobilinogen are of value in diagnosing and detecting pathological processes. In patients with complete and prolonged obstruction of the common bile duct, little if any, bilirubin reaches the gastrointestinal tract, and extremely small amounts of urobilinogen are formed. In this condition, the urobilinogen content of both feces and urine is zero or very low. Such findings are characteristically seen in carcinoma of the head of the pancreas or ampulla of Vater. With calculi in the common bile duct, the obstruction is likely to be incomplete, producing a decrease in fecal urobilinogen but little, if any, increase in urinary excretion.

Individuals with hepatic disease may evidence some abnormality in urobilinogenuria but the results depend on the type and stage at which the patient is observed. Thus, early in the acute phase of infectious hepatitis, normal or almost normal amounts of bilirubin may be excreted into the gastrointestinal tract, and be converted to urobilinogen; the latter is absorbed and presented to the liver via the circulation. However, because of parenchymal cell dysfunction, there is interference with the transfer of the urobilinogen from blood to the bile canaliculi. As a result, the blood levels of the pigments rise, resulting in an enhanced excretion by the kidneys. Under these circumstances, the urinary excretion of urobilinogen may increase to 100 mg. per 24 hours although the usual values found at this stage are between 4 and 10 mg. Later in the course of the disease, especially if liver necrosis or dysfunction is marked,

decreased amounts of bilirubin reach the gastrointestinal tract so that the urinary excretion may even fall to normal amounts. During the stage of recovery from hepatitis, as more bilirubin enters the gut to start the urobilinogen cycle, increased quantities of the latter pigments may again appear in the urine and persist until healing is well advanced.

Patients with hemolytic disease characteristically exhibit fecal urobilinogen excretions that may vary between 500 and 2000 mg. per 24 hours. The urinary excretion of these pigments in such individuals may be normal or may be as high as 40 mg. per 24 hours. There are two possible explanations for this increase: (a) concomitant hepatic dysfunction resulting from the accompanying anoxia interferes with the transfer of urobilinogen; (b) the load of urobilinogen presented to the liver may exceed its maximal transfer capacity, and with rising blood levels, it is excreted by the kidney.

The measurement of the 24-hour excretion of urobilinogen yields results which are considered to be a rather sensitive test for detecting hepatic disease. Increased amounts of the pigments may be found in acute infectious hepatitis, with or without jaundice, pyogenic and amebic abscesses, malaria, and other infectious diseases involving the liver, congestive heart failure, portal cirrhosis (with or without jaundice) biliary cirrhosis and the injuries caused by chemical agents.

In interpreting the results of urinary urobilinogen excretions, several factors deserve mention: (a) antibiotics which alter the gastrointestinal flora may prevent the conversion of bilirubin to urobilinogen; (b) porphobilinogen reacts with Ehrlich's aldehyde reagent to give a color similar to urobilinogen but the latter colored product is chloroform-soluble and the former is not.

Three methods have been suggested for quantitating urinary urobilinogen. These are:

(1) Quantitative 24-hour excretion (Schwartz, Sborov, Watson). This method, although the most tedious, is the one of choice. Precautions must be used in collecting the urine. (2) Simplified quantitative urine Ehrlich reaction. This method measures the urobilinogen excretion in a 2-hour sample of urine collected between 2 and 4 p.m. Although the results are said to be comparable to the 24-hour excretion, examination of the original data on which this is based reveals many divergencies between the 2-hour and 24-hour excretions and is therefore less satisfactory than the 24-hour procedure. (3) Simple dilution test. This procedure is the least satisfactory test and should be abandoned. Like all spot tests, it is subject to the errors of urine dilution. Thus,

1 mg. of urobilinogen may be excreted in four hours in a urinary volume of 120 or 1000 cc. Since the dilution test measures the concentration only and not the total excretion, the different results obtainable on these two urines are obvious.

PLASMA PROTEINS

Since they originate to a considerable extent in the liver, the plasma proteins are frequently altered both qualitatively and quantitatively when hepatic disease is present. Appropriate laboratory tests reflect these changes and thus aid in the diagnosis and differentiation of the various types of hepatic dysfunction. Quantitative changes can be estimated by the determination of serum albumin and globulin (or better, albumin and the globulin subfractions), by chemical or electrophoretic means; a combination of both quantitative and qualitative alterations can be evaluated by the performance of thymol turbidity and cephalin-cholesterol flocculation procedures.

The metabolism of the plasma proteins was discussed in Chapter 7. Suffice it to point out here that any change in the level of the proteins is the resultant of three variables; rate of protein synthesis and release into the plasma, the plasma volume, and the rate of removal of protein from plasma and its degradation. Thus, a decrease in the concentration of serum albumin may result from (a) an increase in plasma volume, (b) a decrease in the amount synthesized daily with a normal rate of utilization, (c) a normal rate of production with an increase in the rate of degradation, and (d) an increased rate of utilization that exceeds an increased rate of production. Studies with isotopes have not clarified all of the alterations in hepatic diseases.

In general, a decrease in functioning hepatic mass, such as occurs with hepatitis, neoplasm, nutritional deficiencies and chronic passive congestion, is associated with a decrease in serum albumin concentration. The fall in albumin level tends to reflect the severity of the disease process. With inflammatory lesions of the liver, serum gamma globulins increase in concentration; in addition, abnormal types appear. Thus, patients with acute viral hepatitis characteristically exhibit some degree of hypoalbuminemia and hyperglobulinemia, the latter especially involving the gamma fraction. It is uncertain whether a decreased synthesis or increased utilization is responsible for the lowering of albumin levels although the sharp fall in concentration during the onset of hepatitis in human volunteers suggests that increased utilization must play a role in the changes seen. The concommitant rise in gamma globulins

is associated with plasma cell proliferation in bone marrow and liver with lymphocytic accumulation in the latter organ; these cells are presumed to be the source of the increased circulating gamma globulins. In addition to the changes in albumin and the gamma globulins, there is a tendency for the alpha globulins to be low and the beta globulins elevated during the acute phase of hepatitis. Serial determinations of the plasma proteins are of aid not only in following the course of acute hepatitis but also in evaluating prognosis; the persistence of a hypoalbuminemia and hypergammaglobulinemia is consistent with either the continuation of the acute process or its progression into a chronic phase. With chronicity, there is a tendency for the alpha globulin fraction to increase in amount.

Individuals with portal cirrhosis and chronic hepatitis show a diminution in the concentration of serum albumin, although on the basis of plasma volume studies, total circulating albumin may be normal; an increase in the beta and gamma fractions is found. As noted above, the alpha fraction may also be increased in concentration.

In contrast to the findings in individuals with acute or chronic hepatitis are the ones observed in those with early obstruction of the common bile duct; in the latter cases, no changes in the concentrations of the plasma proteins are seen. These findings are therefore of value in distinguishing hepatic from posthepatic jaundice, at least in the early stages. With chronicity of the obstructive lesion, an elevation in all the globulin fractions occurs and therefore may mirror the hepatocellular alterations that are the consequence of prolonged obstruction. In xanthomatous biliary cirrhosis and obstructive lesions accompanied by hyperlipemia, the beta fraction of the globulins may be increased in concentration.

It should be emphasized that the changes enumerated are not specific for hepatic dysfunction. However, once a disease entity involving the liver or biliary system has been established, protein changes as followed serially enable one to follow the course of the disease.

Table 8.1 summarizes briefly the quantitative changes in plasma proteins with hepatic and biliary tract disease.

Cephalin-Cholesterol Flocculation

The cephalin-cholesterol flocculation test was devised by Hanger in an attempt to distinguish between obstructive and hepatogenous jaundice. He demonstrated that the serum of patients with liver cell injury produced flocculation of a cephalin-cholesterol emulsion; the serum of nor-

TABLE 8.1

Changes in the Concentration of Plasma Protein Fractions in Hepatic Disorders

Disease Process	Plasma Protein Fractions				
	Albumin	Globulins			
		Total	Alpha	Beta	Gamma
Hepatitis					
Acute	↓	↑	↓	→↑	↑
Chronic	↓	↑	↑	↑	↑
Cirrhosis	↓	↑	↑	↑	↑
Common bile duct obstruction (early)	→	→	→	→	→
Xanthomatous biliary cirrhosis	↓	↑	↑	↑	↑

↑ Indicates increased concentration.
→ Indicates no change in concentration.
↓ Indicates decrease in concentration.

mal individuals and of patients with early biliary obstruction did not cause such an emulsion to flocculate.

Flocculation or lack of flocculation of the cephalin-cholesterol emulsion under the conditions set up by Hanger appears to depend on:

(a) Dilution of the serum. In the procedure, a 1:20 dilution of human serum is used; if a greater dilution is employed, sera from normal individuals as well as from patients with hepatic disease flocculate the emulsion; (b) stabilizing factors. According to Hanger and Moore, there are factors in normal serum albumin and alpha-1-globulins which tend to inhibit the flocculation reaction. These stabilizing factors tend to deteriorate with time after blood is drawn. Within 48 hours after acute hepatic parenchymal injury, the stabilizing factors disappear from the circulating proteins and remain absent until healing is well established. The apparent disappearance of these substances can be accounted for by either a failure of the liver to synthesize them or by the release of inhibitors by the injured liver; (c) changes in the gamma globulins. A positive flocculation does not appear to depend on hypergammaglobulinemia nor on the presence of abnormal gamma globulins; if either of these two conditions are present, however, as they often are in hepatic disease, they tend to favor flocculation.

Individuals with hepatic disease demonstrate a positive cephalin-cholesterol flocculation because there is an increase in flocculating gamma

globulins and a decrease in stabilizing albumin; when a certain degree of imbalance is produced, flocculation occurs.

A slight degree of flocculation is sometimes observed with the serum of normal individuals. A flocculation of zero or 1+ is considered normal.

A flocculation of 2+ to 4+ is abnormal. The cephalin-cholesterol flocculation test is a sensitive indicator of hepatocellular injury and a positive test (2+ to 4+) is seen early in the majority of patients having a damaged liver parenchyma. It is therefore of value in diagnosing hepatitis without jaundice. It should be pointed out, however, that the test is not invariably positive in the course of viral hepatitis; 20 per cent of a group ill with infectious hepatitis did not exhibit a positive flocculation test. The positive flocculation test may or may not extend into the period of recovery from the acute attack; the thymol turbidity procedure (*vide infra*) is apparently of more value during this period. The test is also often positive in chronic liver disease, if necrosis of cells is present; a high percentage of individuals with cirrhosis fall into this category. The test is usually negative in patients with biliary obstruction of short duration. Hence, the test is used not only to detect suspected hepatic dysfunction but also to differentiate hepatocellular jaundice from obstructive types. If the obstructive lesion is not relieved, superimposed cellular injury may cause a positive reaction.

The degree of cephalin-cholesterol flocculation cannot be correlated with any specific disturbance of liver function nor with the extent of hepatic involvement. The test is not positive in all forms of liver dysfunction. It is usually negative in fatty metamorphosis, neoplasms, solitary abscesses, chronic passive congestion, and biliary cirrhosis and cholangitic hepatitis unless there is concurrent involvement of the hepatic parenchyma.

Since a change in the quantitative relationship of albumin and gamma globulins is one of the factors responsible for flocculation, nonhepatic diseases which produce hypoalbuminemia or hypergamma globulinemia (see protein section) may also yield a positive test. Hence flocculations are often seen in conditions associated with increased gamma globulins—kala-azar, rheumatoid arthritis, sarcoid, leprosy, malaria, lymphopathia venereum, etc.—and a positive test cannot be interpreted as indicating liver disease. Further studies should be performed in an effort to evaluate the finding.

A negative flocculation test may be associated with severe liver dis-

ease if serum protein abnormalities do not occur. It is best to employ the cephalin-cholesterol test in conjunction with the thymol turbidity and other hepatic tests in order to obtain more meaningful data. When employed serially, the test is helpful in following the course of hepatic disease.

The method of Hanger, observing a number of precautions listed in Hawk, Oser, and Summerson has continued to be used with few modifications.

Thymol Turbidity

The thymol turbidity test (Maclagan, 1944) is another empirical liver function test that was introduced as an aid in demonstrating hepatic cellular dysfunction. Its use stems from the observation that some degree of turbidity is produced when serum is mixed with a saturated solution of thymol. The amount of turbidity can be measured quantitatively either in a photoelectric colorimeter or by comparison with artificial standards. Under the conditions of the test, sera from patients with hepatitis cause a greater degree of turbidity than sera from normal individuals.

In general, it appears that the factors responsible for the degree of turbidity are correlated with qualitative and quantitative changes in the serum albumin and gamma globulin fractions (Kunkel and Hoagland; Maclegan, 1948). Albumin inhibits the development of turbidity while gamma globulins promote its formation, although there is no direct correlation between the degree of turbidity and the elevation in gamma globulins. In contrast to the cephalin-cholesterol flocculation test, the abnormal and increased amounts of the gamma fraction which are found in hepatic disease play a larger role in producing an abnormal result. There is also evidence that some of the lipids associated with the beta globulins of serum increase turbidity formation (Cohen and Thompson). The sera obtained from lipemic individuals with no manifestation of hepatic dysfunction usually have a high turbidity reading. Other factors unknown at present may also enter into the reaction.

Using the colloidal glass standards of Reinhold, normal turbidity does not exceed 5 units. Values above this are most frequently associated with hepatic dysfunction.

The test has its greatest usefulness in following the course of an acute hepatitis, during which serial determinations should be employed, though it may not reveal abnormal values until later in the disease than the cephalin-cholesterol flocculation procedure. Since the thymol turbidity is often the last test to return to normal, it may aid in demonstrating

residual hepatic cellular damage. When the thymol test is employed in conjunction with other liver function tests, it is also of use in the differential diagnosis of jaundice. It is usually negative in biliary obstruction and positive in hepatocellular disease.

For the most part, there is a rather high degree of correlation between results obtained by the cephalin-cholesterol flocculation and thymol turbidity tests. The thymol test has the advantage of being readily and accurately quantitated turbidimetrically by means of a photoelectric colorimeter whereas cephalin-cholesterol flocculations are rather crudely graded from 0 to 4 plus.

The thymol turbidity procedure suffers from the same lack of specificity as does the cephalin-cholesterol. Neither measures any specific hepatic function and each may give positive values in non-hepatic disease if requisite alterations in the serum protein pattern are present. A positive result is *not* specific evidence of liver disease and should be confirmed by other hepatic tests. In addition, there appears to be no good correlation in liver disease between the degree of turbidity and the severity of hepatic involvement. Thus, the greatest turbidity may be found in severe liver damage secondary to biliary obstruction or cirrhosis; part of the turbidity in these cases may be the resultant of the increased serum lipids (lipoproteins) which react with the thymol reagent to produce a turbidity indistinguishable from that produced by the protein changes. Increased serum gamma globulins, associated with multiple myeloma, lymphogranuloma venereum, sarcoidosis and parasitic infection, may cause an abnormal thymol turbidity even though there may be no hepatic involvement. By contrast, individuals with nutritional cirrhosis and fatty livers may have elevated serum gamma globulin concentrations with a normal thymol turbidity. Individuals with cirrhosis may or may not have an abnormal test.

Neither the thymol turbidity nor the cephalin-cholesterol flocculation test should be employed alone as a "screening" or as a differential diagnostic procedure. More information may be obtained by using both methods rather than by employing either alone. This is due to the fact that the results of each test will vary with the disease process. For differentiation between the several types of jaundice, the most useful information is obtained by combining these with other tests of hepatic function.

Thymol Flocculation (Neefe)

After measuring the turbidity, the serum-thymol mixture is allowed to stand overnight. In some cases of hepatitis, a flocculation is observed

but the factors responsible for this are not known; the presence of an abnormal serum protein which may affect flocculation is suspected. The precipitate consists of a protein-thymol-phospholipid complex and the degree of flocculation is graded from 0 to 4+. In patients with hepatocellular disease, an abnormal thymol flocculation test does not occur as frequently as do abnormal results with thymol turbidity or cephalin-cholesterol flocculation procedures. The finding of a normal thymol turbidity and an abnormal thymol flocculation may indicate a chronic low-grade hepatitis. In those cases where the thymol flocculation is abnormal, however, the positive finding may persist after the two other tests have reverted to normal.

CHOLESTEROL

The liver plays a major role in the homeostasis of plasma cholesterol (for full discussion of cholesterol metabolism, see Chapter 7, Blood Chemistry) in that hepatic cells are the source of this serum lipid as well as an active site for its degradation and excretion. Furthermore, the maintenance of the normal proportion of free and esterified cholesterol is dependent on normal hepatocellular enzymatic activities. Since hepatic cellular dysfunction as a result of disease processes can often be correlated with changes in total serum cholesterol and absolute amounts of the free and esterified form, the estimation of the level of cholesterol and its fractions in serum serves as a means of evaluating hepatic injury and as an aid in the differential diagnosis of jaundice. In general, it should be emphasized that the cholesterol-cholesterol ester fractionation is not considered a very sensitive test for the presence (or absence) of a pathologic process involving the liver; apparently widespread hepatic disease must be present before it is reflected by the results of the test.

With the onset of jaundice in acute hepatitis, the concentration of total serum cholesterol characteristically diminishes, chiefly because the level of esterified cholesterol decreases in absolute amount (and hence in percentage of the total). There is a tendency for the level of total serum cholesterol to vary inversely with the extent of liver injury, i.e., the greater the injury, the lower the concentration of total cholesterol. Because of the wide range of normal values, a decrease in the cholesterol concentration of a particular patient may not be apparent in the first determination if it lies above the lower limit of normal; with recovery, however, a rise in the serum cholesterol level to that characteristic of his healthy state will demonstrate the depression produced by the dis-

ease. The esterified fraction may decline to undetectable amounts but this finding by itself appears to have no prognostic significance; of eight such cases observed in the authors' laboratory, with cholesterol esters of zero, only two deaths occurred while those who recovered evidenced no residua of hepatic disease. During recovery from the acute disease, esterified cholesterol, and often the free fraction, increases in amount so that elevated concentrations of total cholesterol may be observed until normal function is regained. The portal cirrhosis following chronic hepatitis or malnutrition is also characterized by low serum total and esterified cholesterol.

In extrahepatic obstruction of the common bile duct, a moderate to large increase in the concentration of serum total cholesterol occurs, depending on the etiology and duration of the obstruction. Under these conditions, the increased cholesterol levels seen are mainly in the free fraction; the esterified cholesterol is either normal or increased in absolute amount but decreased when expressed as a percentage of the total. These differences in the behavior of the fractions of serum cholesterol in hepatic and posthepatic diseases are summarized in Table 8.2.

The cholesterol-cholesterol ester determinations are indicated in two types of jaundiced individuals. One group consists of patients with acute hepatitis in whom flocculation tests are maximally positive, bearing in mind that the cholesterol results frequently have no prognostic significance. Patients with extrahepatic biliary obstruction should likewise be followed with serial cholesterol fractionations since the results may aid in evaluating the development of secondary hepatocellular involvement. For these reasons, the determination of serum cholesterol

TABLE 8.2

Changes in Serum Cholesterol Concentration in Several Types of Liver Diseases

Disease	Cholesterol			
	Total	Free	Esterified	
	mg./100 ml.	*mg./100 ml.*	*mg/100 ml.*	*% of total*
Hepatitis, acute	→↓	→↓	↓	↓
Hepatitis, chronic	→↓	→↓	↓	↓
Posthepatic biliary obstruction	→↑	→↑	→↑	→↓
Biliary cirrhosis	↑	↑	↑	→↓

↑ Indicates increased concentration.
→ Indicates no change in concentration.
↓ Indicates decrease in concentration.

and its esters appears indicated whenever jaundice is present. On the other hand, when liver dysfunction is minimal, the BSP and flocculation tests are the procedures of choice since they are simpler, more sensitive to disturbances in hepatic activity and less costly to perform than the estimation of cholesterol and its esters. In similar fashion, the determination of the serum phospholipid concentration is of more value than that of cholesterol as an aid in establishing the presence of xanthomatous biliary cirrhosis. Although the cholesterol level is characteristically increased in this type of hepatic dysfunction, the serum concentration of phospholipid is elevated to a relatively greater degree and may reach concentrations of over 3000 mg. per 100 ml.

Other disease states, e.g., hypo- and hyperthyroidism, the anemias, uncontrolled diabetes, nephrosis, and sometimes atherosclerosis, which are associated with altered concentrations of serum cholesterol, must be considered when using the cholesterol determination as a hepatic test.

ALKALINE PHOSPHATASE

Alkaline phosphatase, an enzyme present in osteoblasts, liver, intestinal mucosa, kidney, plasma and other tissues, is capable of hydrolyzing monophosphate esters at pH 9 to 10 (see Chapter 7, Blood Chemistry). Although the main source of the serum enzyme in normal conditions may be the osteoblasts, the liver participates in the regulation of its level (actually estimated by measuring phosphatase activity) by slowly extracting this enzyme from the blood and excreting it into bile. When the bile ducts are obstructed by extrahepatic or intrahepatic processes, the serum activity of the enzyme rises for reasons unknown. As a consequence of disorders affecting the parenchyma, it is felt that hepatic cells may elaborate and transfer to plasma excessive amounts of alkaline phosphatase, especially when the cells are regenerating.

A marked elevation in serum alkaline phosphatase activity (above 10 or 15 Bodansky units) commonly occurs in patients with extrahepatic biliary obstruction and inflammatory disease of the bile ducts (cholangiolitic hepatitis). Similar values may be found occasionally in individuals with cirrhosis or metastic carcinoma of the liver, even in the absence of jaundice. Congenital biliary atresia, however, is the one type of extrahepatic biliary obstruction that exhibits normal values for serum alkaline phosphatase activity. While a moderate increase in the serum enzyme activity (4 to 10 Bodansky units) is the common finding in patients with hepatocellular injury, comparable values may also be seen in those with incomplete extrahepatic obstruction, hepatic tumor

metastases, liver abscess or passive congestion of the liver. From the above, it may be seen that this test is unreliable when used alone for differentiating between surgical (extrahepatic) and medical (intrahepatic) jaundice. When coupled with other hepatic tests, particularly the flocculation or thymol turbidity procedures, its usefulness is increased. The finding of a high alkaline phosphatase activity with a negative cephalin-cholesterol flocculation and a normal thymol turbidity favors the presence of extrahepatic biliary obstruction. In contrast to the above, the finding of a moderately elevated alkaline phosphatase activity coupled with a positive cephalin-cholesterol flocculation or a thymol turbidity exceeding 5 units suggests the presence of parenchymal injury. Since a marked increase in serum phosphatase activity is more difficult to evaluate from the standpoint of etiology, the finding of a low or moderate level of enzyme activity is of more diagnostic significance.

The estimation of alkaline phosphatase activity may also be used for prognosis in severe liver disease. With overwhelming liver necrosis and a rising serum bilirubin level, the finding of a low or falling serum alkaline phosphatase has grave prognostic significance that is attributed to the lack of regeneration of hepatic cells with their rich source of enzymes.

In the absence of excessive osteoblastic activity of bone, the finding of an increase in the activity of serum alkaline phosphatase suggests that studies be instituted to determine whether hepatic or posthepatic disease be present. The test is not considered to be a sensitive index of hepatic dysfunction; on the other hand, certain types of lesions that can not be detected by other methods, such as metastatic carcinoma and hepatic abscesses, are often first revealed by an elevation in the activity of the enzyme. There are additional difficulties in interpreting this test when performed on children because of the high and variable serum alkaline phosphatase activity that is normally present.

BROMSULPHALEIN TEST

Bromsulphalein (BSP, sodium phenoltetrabromophthalein sulfonate, sulfobromophthalein) is one of a number of dyes which, after intravenous injection, is removed from plasma by liver (Rosenthal and White). Although it was once believed that Kupfer cells removed the dye from the plasma and transferred it to the polygonal cells which in turn excreted it into the biliary canaliculi, there is now good evidence that the Kupfer cells have no role in the process (Nendeloff). BSP is probably removed from plasma directly by the polygonal cells, transferred to bile and eliminated in the feces. In this manner, the liver removes about 80

per cent of the injected dye. The ability of the liver to remove the dye has made it the basis of a test measuring hepatic excretory capacity. During the process of excretion into bile, there is evidence that some chemical changes occur in the molecule of the dye. The significance of these changes are not understood. Extrahepatic tissues other than the viscera remove some 10 to 20 per cent of the injected BSP; studies with S^{35} labelled material implicates skeletal muscle as the major extrahepatic site for extraction of the dye.

Many proposals as to methodology for measuring the maximal ability of the liver to transfer BSP have been made in the interest of increasing the sensitivity of the test. In general, all suggestions employ the principle of estimating the amount of dye remaining in the circulating plasma at some time interval(s) after a stipulated dose has been injected. At present, 5 mg. of the dye per kg. body weight appears to be the most satisfactory amount for testing the maximal transfer capacity of the liver. The time after the injection at which bloods are taken for estimation of circulating BSP is not too critical, provided standards of normal are known; both 30 and 45 minutes have been suggested. With the 30 minute sample less than 1 mg. per 100 ml. and with the 45 minute sample less than 0.4 mg. per 100 ml. of serum are circulating normally. Some laboratories measure the disappearance rate of BSP by means of multiple samples collected at specified time intervals after the injection.

Regardless of the method employed, a decrease in the capacity to remove bromsulphalein from plasma may be caused by one or more of the following abnormalities:

1. Decresed hepatic blood flow: If the blood flow to the liver is decreased, as in congestive heart failure, less BSP will be presented to the liver per minute and less will be removed. If the circulatory impairment is marked, an otherwise normal excretory capacity may be unable to effect a normal clearance.
2. Hepatic cell damage: The polygonal cells may be damaged by disease, thus deranging the BSP transfer mechanism. Such a decrease in total transfer capacity for the dye may be the result of a minor degree of involvement of all the polygonal cells or a marked degree of derangement of only a portion. Therefore, the BSP test is most appropriately used to detect suspected hepatic dysfunction or to evaluate the status of the liver after an attack of hepatitis. In the latter condition, it helps to distinguish between recovery and progression of the disease to a chronic phase. The procedure is a very valuable adjuvant in assaying for cirrhosis. In upper gastro-

intestinal hemorrhage, the test aids in differentiating a bleeding peptic ulcer from cirrhosis with esophageal varices. It is the only test capable of detecting fatty metamorphosis of the liver but does not do so consistently.

The test may also yield valuable information when performed in patients with toxemias of pregnancy. When there is retention of BSP in individuals with cholecystitis or cholelithiasis, it may indicate that there is also an ascending cholangiolitis. Patients with primary or metastatic carcinoma to the liver may also show abnormal retention if the process has involved a sufficient amount of the liver. When performed serially in these types of pathology, BSP serves both as a diagnostic and prognostic procedure.

3. Biliary obstruction: Even with a normal blood flow and enzyme transfer system, there can be no hepatic removal in the presence of complete biliary obstruction. The dye will remain in the plasma until extrahepatic sites clear it.

It is obvious that the bromsulphalein test will be of most use in evaluating hepatic cellular status in the absence of hyperbilirubinemia. An evaluated serum bilirubin is prima facie evidence of a decreased hepatic excretory capacity, if excessive hemolysis can be eliminated as the cause. In the presence of jaundice, there is no reason for performing the BSP test. Although there is some evidence that in jaundiced patients the degree of BSP retention may parallel the disease process, other tests of hepatic function such as serial serum bilirubin levels, serum cholesterol and esters, and quantitative urinary urobilinogen determinations are more valuable laboratory procedures for following the course of the disorder.

The procedure is one of the most sensitive and reliable for estimating total functioning hepatic mass. However, as noted above, it is not specific for metabolic transfer activity, being depressed by extrahepatic factors.

The test is performed most simply by injecting 5 mg. of the dye per kg. body weight (or of the ideal body weight, see Hawk, Oser and Summerson) and measuring the amount of dye remaining in serum at the end of 45 minutes by the method of Bradley and Inglefinger.

APPLICATION OF HEPATIC TESTS

It was pointed out in the introduction to this chapter, that hepatic tests are not always capable of indicating the presence (or absence) of disease. The chemical tests described should be considered as adjuvants

to the clinical examination of the patient in conjunction with the experience and judgement of the physician. X-rays, liver biopies, and biliary drainage also have a role in the evaluation of the state of the patient. Despite the use of all procedures, there will still be patients who remain diagnostic enigmas. Thus, not only may there be a poor correlation between hepatic architecture and the results of laboratory procedures, but the various procedures may fail to implicate a particular disease process. This situation may arise from the fact that each test reflects a different facet of the overall metabolic integrations taking place in the liver.

Table 8.3 summarizes the expected results obtained with hepatic tests in the various types of jaundice; in Table 8.4 we have given our recom-

TABLE 8.3

The Usual Results Obtained with Hepatic Tests in the Various Types of Jaundice and Hepatic Disease

Hepatic Function Test	Types of Disease				
	Prehepatic jaundice	Hepatic disease		Posthepatic jaundice	
		Without jaundice	With jaundice	Neoplasm	Calculus
Serum bilirubin	↑	→	↑	↑	↑
Urine bilirubin	—	+	+	+	+
24-hour urinary urobilinogen	→↑	↑	↑	↓	→↑
Total cholesterol	↓	→↓	→↓	→↑	→↑
Cholesterol esters					
mg. 100 ml	↓	→↓	→↓	→↑	→↑
% of total	→↓	↓	↓	→↓	→↓
Thymol turbidity		+	+	−	−
Thymol flocculation		+?	+?	−	−
Cephalin-cholesterol flocculation		+	+	−	−
Alkaline phosphatase		→↑	→↑	↑	↑
Changes in					
Serum albumin		↓	↓	→	→
Serum globulin		↑	↑	→	→
BSP test		+*			

* The only type of hepatic disease in which the test is indicated.

↑ Indicates increased concentration.

→ Indicates no change in concentration.

↓ Indicates decrease in concentration.

— Indicates normal reactions or a negative test.

\+ Indicates an abnormal reaction or a positive test.

TABLE 8.4

Recommended Selection of Hepatic Tests

Hepatic Function Test	Detection of Liver Disease without Jaundice	Detection of Residual Hepatic Injury after Recovery from Hepatitis, etc.	Following Patients with Hepatic Disease	Following Surgical Patients with Biliary Tract Disease
Serum bilirubin	R	R	R	R
Urine bilirubin	R	—	—	—
24-hour urinary urobilinogen	R	R	S	—
Cholesterol and esters	—	—	S	—
Thymol turbidity and flocculation	R	R	R	—
Cephalin-cholesterol flocculation	R	R	R	—
Alkaline phosphatase	R	—	—	R
Albumin and globulin fractions	—	R	R	R
BSP	R	R	—	—

R, tests useful.
S, tests valuable under special conditions.
—, little information gained by the performance of this test.

mendations on the use of these tests. It will be noticed that not every test is indicated in all jaundiced individuals. While more information may be obtained as more tests are performed, a point of diminishing returns is soon reached. Since the serial performance of many tests is often economically impossible, judgement is required not only in the choice of test but also the frequency of repetition. Although we indicated multiple tests as being useful in following the course of hepatic disorders, it does not mean that all of the tests should be used indiscriminately. If the result of a test can have no influence upon the treatment prescribed during the acute phase of a disease, there is little value in the performance of such a determination. In this situation, the measurement of serum bilirubin alone may suffice until the chronic or recovery stage is reached.

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CHAPTER 9

THE ASSAY OF CHEMOTHERAPEUTIC AND ANTIBIOTIC AGENTS

By Albert Milzer

GENERAL CONSIDERATIONS

The term "antibiotic" was suggested by Waksman *et al.* (1942) for substances elaborated by microorganisms that have the ability to inhibit the growth of other species of bacteria, especially pathogens. Antibiotics are derived chiefly from three sources: (1) *molds*, e.g., penicillin, (2) *actinomycetes*, e.g., streptomycin, chlortetracycline, chloramphenicol and oxytetracycline and (3) *bacteria*, of which bacitracin and polymyxin are examples. The concept of antagonistic action among microorganisms is not new. As early as 1877 Pasteur and Joubert observed the antagonism between *Bacillus anthracis* and other bacteria in cultures. The first antibiotic described was pyocyanase, produced by *Pseudomonas aeruginosa* (Emmerich *et al.*, 1899). Penicillin, as is well known, was discovered by Fleming in 1929, but its possibilities were not appreciated until the work of Chain *et al.*, in 1940. Dubos and Hotchkiss (1941) isolated tyrothricin from *Bacillus brevis* from which they separated two polypeptides, gramicidin and tyrocidine. This discovery stimulated further work so that about 300 antibiotics have been described thus far (Welch, 1954). Unfortunately, most of them are too toxic and only about a dozen can be used clinically.

Chemotherapeutic substances refer to synthetic drugs such as the arsenical and the sulfonamide compounds. Chemotherapy was initiated by the pioneer studies of Ehrlich starting in 1905 which culminated in the use of arsphenamine for the therapy of syphilis and a number of other spirochaetal infections. The discussion in this chapter is limited to a consideration of the assay and clinical use of sulfa drugs. Chemotherapy of bacterial infections with sulfa drugs started in 1935 with Domagk's report that prontosil would cure streptococcal infections in mice. It was soon shown that p-amino-benzene sulfonamide was the active principle of prontosil. Since then a number of effective sulfonamide compounds have been synthesized with increased antibacterial activity and decreased toxicity.

The sulfa drugs are primarily bacteriostatic rather than bactericidal in the usual therapeutic concentrations. They have no effect unless cell division is taking place. The current hypothesis is that the sulfa drugs

inhibit growth by competition for a special enzyme system associated with an essential metabolite, p-amino-benzoic acid (Woods, 1940; Fildes, 1940). The addition of p-amino-benzoic acid will revive the growth of bacteria exposed to sulfa drugs both in vitro and in vivo. On the other hand, the mode of action of penicillin and the other antibiotics in terms of cellular metabolism is unknown. Penicillin acts more rapidly and is more bactericidal than the sulfa drugs. In contrast to the latter, penicillin is lethal to cells that are undergoing metabolism although no actual cell division is taking place. The morphology of inhibited cells shows a tendency toward giant cell formation and may ultimately undergo lysis (Gardner, 1940).

The multiplicity of antibiotic and chemotherapeutic agents available today makes it imperative that a precise etiologic diagnosis be established for optimum results in therapy. The use of improved culture media for bacterial infections such as thioglycollate media in recent years has resulted in a greater percentage of positives as well as more rapid isolation of the etiologic organisms. The use of sternal marrow cultures has also served to increase the number of positive results.

Whenever possible, specimens for culture and sensitivity tests should be taken *before* starting antibacterial therapy because of the difficulty in isolating and identifying bacteria exposed to these drugs. The incubation period for growth is often prolonged and alteration in morphologic and physiologic characteristics may occur which hampers identification. The laboratory may be compelled to subculture for several generations before the organisms are sufficiently typical to permit identification.

In vitro sensitivity tests for many bacterial infections are now available so that the clinician may select the antibiotic of choice when routine therapy fails instead of resorting to the costly and dangerous trial and error method. Furthermore, the results of sensitivity tests may also serve as a guide to the dosage of drug which should be employed. Serum level assays may be done in selected cases in order to check whether adequate therapy is being given. It has now been amply demonstrated that there is some correlation between in vitro results and clinical response with certain exceptions (Broom *et al.*, 1953; Weil *et al.*, 1953). The screen testing of bacterial cultures against a battery of antibiotics is rapidly becoming a routine procedure in many diagnostic laboratories.

BACTERIAL RESISTANCE

The problem of the development of bacterial resistance to the chemotherapeutic and antibiotic agents is becoming of increasing importance.

The improper use and abuse of these drugs is producing numerous resistant strains which obviously will reduce their value in therapy and could conceivably give rise to uncontrollable epidemics in the future. There are many reports in the literature concerning the development of both in vitro and in vivo resistance of various species and strains of bacteria to the sulfa drugs, penicillin and streptomycin. For instance, during World War II it was shown that military populations undergoing mass chemoprophylaxis with small doses of sulfadiazine (0.5 to 1 gram daily) were infected with Group A hemolytic streptococci which readily became resistant to this drug (Epidemiology Unit No. 22, 1945; Damrosch, 1946). Similarly, increased resistance of the bacterial strain has been encountered during sulfa therapy of Shigella dysentery, gonorrhea, pneumococcus and staphylococcus infections.

The incidence of penicillin-resistant strains of *Staphylococcus aureus* both in England (Barber *et al.*, 1948) and in the United States (Romansky, 1951; Spink, 1951) is increasing at an alarming rate, especially on hospital wards. Barber *et al.* (1948) in summarizing the increased incidence of penicillin-resistant strains state that they found 14.1 per cent resistant in 1946, 38 per cent in 1947 and 59 per cent in 1948. Spink (1951) reports similar findings and presents evidence that many clinical failures with penicillin can be attributed to penicillin-resistant strains. Romansky (1951) found that 55 per cent of the strains of nonhemolytic streptococci and 98 per cent of the *Streptococcus fecalis* strains which he isolated from infectious processes during 1949 showed varying degrees of penicillin-resistance. Strains of hemolytic streptococci (Group A), pneumococci and meningococci which he tested in the same year were not resistant. These observations parallel our own experience. In addition we have encountered resistant strains of alpha hemolytic streptococci isolated from patients with subacute bacterial endocarditis (Schlichter *et al.*, 1949). Welch (1954) has emphasized the fact that more recent studies made of out-patients and normal subjects indicate that the incidence of penicillin-resistant coagulase positive staphylococci in hospital populations has been considerably greater than that found in nonhospitalized individuals. Apparently cross-infection is a very important cause of infections by antibiotic-resistant staphylococci in the hospital. Prevention must depend on proper hospital hygiene and asepsis. Several years of clinical evaluation indicate that penicillin resistance has not been a problem in Group A hemolytic streptococcus, gonococcus, meningococcus or pneumococcus infections.

The rapid development of *maximum* resistance to streptomycin is

known to occur during the course of therapy of a number of infections. Bacteria that develop resistance to streptomycin are usually also resistant to dihydrostreptomycin. Development of streptomycin resistance has been reported in urinary tract infections due to a number of different Gram negative bacilli (Finland *et al.*, 1946), influenzal meningitis (Alexander *et al.*, 1947) and tuberculosis (Muschenheim, 1947). Romansky (1951) states that 33 per cent of *Escherichia coli* strains, 4 per cent of *Aerobacter aerogenes* strains, 70 per cent of *Proteus* strains, 77 per cent of *Pseudomonas aeruginosa* strains, 33 per cent of nonhemolytic streptococcus strains, and 77 per cent of *Streptococcus fecalis* strains which he isolated from patients in 1949 were streptomycin-resistant. Some clinicians are of the opinion that the use of streptomycin should be restricted because of the relative ease with which maximum resistance is developed

Recent studies (Welch, 1954) indicate that bacteria are also able to acquire resistance to some of the newer antibiotics including chlortetracycline, oxytetracycline, tetracycline, erythromycin, etc. Apparently resistance to the latter occurs much less readily and to a lesser degree thus far than in previous studies with the sulfa drugs and streptomycin. Nevertheless, since the tendency of bacteria to acquire resistance does exist the importance of using the newer antibiotics intelligently and only when indicated in the proper dosage cannot be overemphasized. Otherwise, resistant strains may appear and result in infections refractory to chemotherapy.

The exact mechanism of the development of increased bacterial resistance is not clear. It has been suggested that spontaneous mutation or the presence of an adaptive enzyme may account for this phenomenon. In any event, it is known that all bacterial populations even in a single colony are heterogenous and that a few cells relatively more resistant than the majority are usually present. Alexander (1949a) believes that there is ample proof for Demerec's theory that bacteria resistant to the action of antibacterial agents arise spontaneously as the result of mutation. The gradual development of resistance through many subcultures is believed to represent successive mutations. She further states that there is now no data to support the hypothesis that resistance is the result of specific adaptation of the organism in response to the presence of an antibacterial agent. Spink *et al.* (1947) conclude that the resistance of staphylococci to penicillin at least is related to the speed and potency of penicillinase production. Penicillinase is an enzyme which destroys penicillin.

BACTERIAL SENSITIVITY TESTS

Although identification of the etiologic organism frequently gives adequate information for the initiation of antibacterial therapy, it is occasionally necessary or advisable to check bacterial sensitivity because it may vary considerably among strains of the same species. It is especially important to determine the in vitro sensitivity of strains of coagulase-positive staphylococci from all infectious processes, and alpha hemolytic streptococci isolated from patients with subacute bacterial endocarditis. I have already mentioned that these species of bacteria are notorious for their ability to develop increased resistance in vivo. Other species except *enterococci*, *Pseudomonas aeruginosa* and possibly *Proteus* are not tested routinely unless they continue to be cultured after the beginning of therapy. Sensitivity tests may on occasion be of aid in the preoperative preparation of surgical patients. The value of sterilizing the bacterial flora of the bronchi before lung resection is well established. The bacterial sensitivity dose is defined as the least amount of antibacterial drug necessary to inhibit growth of the infecting organism under certain specified conditions.

Two principal methods for testing bacterial sensitivity are in routine use by many hospital laboratories at present. The first is the serial tube or plate dilution method (Kolmer *et al.*, 1951) and the second is the filter paper disc method (Vincent *et al.*, 1944; Bondi, *et al.*, 1947). There are several modifications of these two types of sensitivity tests because there is no general agreement as to the best method for determining bacterial resistance. The present discussion will be confined primarily to technics which have been used successfully at Michael Reese Hospital during the past few years for routine sensitivity tests. The applicable technics will be described under each antibacterial agent discussed. The reader is referred to publications of Kolmer *et al.* (1951) and Welch (1948) for details of technics.

Tube Dilution Method. This method has the advantage of direct measurement of sensitivity in terms of drug concentration and is the most accurate. The necessary pure culture isolations, however, require time. Essentially, this technic consists of adding graded amounts of antibiotic to suitable broth culture media. The tubes are then inoculated with a measured amount of the test organism and incubated at 37°C. A control tube without antibiotic must always be included to check the viability of the culture. A second control tube with antibiotic seeded with an organism of known sensitivity should be made simultaneously in order to check possible deterioration of the antibiotic.

Results are reported as the lowest concentration, in units or micrograms per ml., which inhibits growth completely.

Plate Dilution Method. This is a modified tube dilution technic carried out by mixing varying concentrations of the antibiotic with melted agar and pouring plates. A number of cultures can be inoculated on each plate, and the results of several sensitivity tests determined simultaneously. Frank *et al.* (1950) recommend the plate dilution method for use in comparative sensitivity tests when a number of strains of the same organism are to be tested with all of the antibiotics simultaneously. In many comparative tests, they found that there was close agreement when the same organisms were tested simultaneously by this and the tube dilution method. They further state that when differences did occur, the end point in the plate dilution was usually at a concentration that was twice that obtained by the tube dilution technic. Schatten *et al.* (1953) found the agar dilution and tube dilution methods of determining bacterial sensitivity equally reliable for penicillin, bacitracin, streptomycin, chloramphenicol, and oxytetracycline. Plates containing these latter five antibiotics could be stored for as long as four weeks in the refrigerator at 4°C. without loss of potency, while chlortetracycline containing plates could be stored for only three weeks. In unpublished studies the writer's laboratory has found the agar dilution method equivalent to the tube dilution technic for polymyxin sensitivity testing.

Filter Paper or Tablet Disc Method. This technic consists of placing discs of filter paper or tablets about 6.5 mm. in diameter on the surface of an agar plate previously streaked with the test organism. The filter paper or tablet contains varying concentrations of antibiotic to be tested. The antibiotic inhibits growth of susceptible organisms by diffusion into the agar. This results in a clear zone surrounding the disc; the diameter of the zone of inhibition is believed to be proportional to the amount of antibiotic substance present. As many as 2 or 3 antibiotics may be tested simultaneously on one agar plate (see Fig. 9.1). Filter paper discs impregnated with various antibiotics are available commercially from the Difco Laboratories in Detroit, Michigan and the American Hospital Supply Corp. (Desi-Discs) of Evanston, Ill. Dia-Disc tablets can be purchased from the Commercial Solvents Corp. in New York City.

The filter paper disc method is applicable to mixtures of bacteria as well as to pure cultures. It can be carried out on the original streak plate used for isolation and give information relative to sensitivity to several antibiotics even before isolation is completed. More exact titrations should be carried out after pure cultures have been obtained. The

advantages are the simplicity of the technic and the fact that a minimum of equipment is needed. We have confirmed Howe's finding (1950) that the serial tube technic is a more accurate test for assay than the filter paper disc method. Comparative studies indicate that the drug sensitivity levels of bacteria tested by the filter paper disc method are usually several serial dilutions lower (more resistant) than levels obtained by the tube dilution method. This method of assay can only give approximate qualitative results and interpretation of sensitivity in terms of millimeters of growth inhibition is fallacious. The paper disc method is especially of value as a screening method for weeding out highly resistant strains. It should be standardized initially by the tube dilution method (Lind *et al.*, 1952). Schatten *et al.* (1953) state that the agar diffusion method of sensitivity determination (filter paper disc) is not fully reliable as shown by the poor correlation of the results obtained by that method and the tube dilution method of testing with streptomycin, chloramphenicol, oxytetracycline and chlortetracycline. On the other hand, there was fair agreement in the case of penicillin and bacitracin.

Braude *et al.* (1952) report good agreement between commercially prepared tablet disc (Dia-Disc) and a tube-dilution test for determining bacterial sensitivity to chloramphenicol, oxytetracycline and penicillin. The disc method was unsatisfactory for determining sensitivity to chlortetracycline probably largely due to the rapid deterioration of this antibiotic.

SULFA DRUGS

It is essential to employ a suitable medium for in vitro sulfa drug sensitivity tests because of the presence of sulfa-inhibitors in many types of culture media. Goodale *et al.* (1943) have developed a culture technic for testing the resistance of *N. gonorrhoeae* to sulfonamides by adding various concentrations of sulfathiazole to Bacto-Mueller Hinton Medium. Other substances antagonistic to the sulfonamides include purulent exudates and peptones. The antagonist in these substances is believed to be p-aminobenzoic acid or its analogues. Harper and Cawston (1945) state that the sulfa-inhibiting substances in nutrient agar can be neutralized by the addition of 5 to 10 per cent horse blood.

Other factors which influence the results include the size of the inoculum, the bacterial species, the rate of growth and the production of sulfa-inhibiting substances during the growth of the test bacteria.

The determination of sulfonamide levels in blood or other body fluid is a chemical procedure and is discussed in the chapter on blood chem-

istry. Occasionally, the laboratory is requested to carry out sulfonamid sensitivity tests, especially if the etiologic organism is resistant to man of the antibiotics or the patient is unable to tolerate the drug of choic The following unpublished screen test is used at Michael Reese Hospita to determine in vitro sensitivity to sulfadiazine, sulfathiazole and gantri sin (3,4,dimethyl-5-sulfanilamido-isoxazole):

1. Plain or blood agar plates are prepared from Bacto-Mueller Hinto Medium containing a final concentration of 5 or 10 mg. per cent o the soluble salt of the test sulfa drugs. This concentration cor responds to an average effective blood level (Price *et al.*, 1951).
2. Seed the surface of the sulfa plates together with a control plat with 0.05 ml. of a 1:1000 or 1:10,000 dilution depending on tur bidity of growth of a 6 hour broth culture of the test organism.
3. Incubate the plates at 37°C. for 24 hours, and note the degree o inhibition by comparing the amount of growth on the sulfa con taining plates with the control plate.
4. Usually there is some correlation between complete inhibition of growth by a given sulfa drug and therapeutic effect.

In our experience the screen test described often shows a fairly good correlation between complete inhibition of growth by a given sulfa drug and therapeutic effect when given in the dose recommended by the manufacturer. This test is applicable to the soluble salts of sulfadiazine, sulfathiazole and to the drug gantrisin (3,4,dimethyl-5-sulfanilamido-isoxazole), but not to the insoluble sulfa drugs, sulfathalidine or succinylsulfathiazole (sulfasuxidine). Tests with sulfamerazine are not done because this drug is therapeutically equivalent to sulfadiazine and these two drugs are interchangeable when the blood level concentration is the same. In general we have found sulfathiazole more potent than sulfadiazine or gantrisin in the screen test, but the latter are preferable for treatment because they are less toxic.

The sulfonamide screen test is chiefly applicable to strains of certain Gram negative bacteria such as *Proteus* spp., *A. aerogenes*, *E. coli* and *Ps. aeruginosa* that are resistant to many of the antibiotics or if the drug of choice is toxic to the patient. Routine use of the screen test is not indicated in other infections as beta hemolytic streptococcal, pneumococcal or meningococcic meningitis that either respond readily to the usual dosage or are preferably treated with penicillin or other less toxic antibiotics (see Tables 9.5 and 9.6).

We have isolated occasional strains of *Ps. aeruginosa*, *A. aerogenes* and *Proteus* spp. from patients that were resistant to all of the antibi-

tics listed in Tables 9.1 and 9.2 except polymyxin and whose growth was completely inhibited by one of the sulfa drugs in the screen test. Such infections subsequently responded to treatment with the indicated sulfa drug, although previous therapy with the various antibiotics was unsuccessful (Milzer *et. al.*, 1952). We and others (Carroll *et al.*, 1950) have found gantrisin especially valuable in the treatment of urinary tract infections due to antibiotic resistant strains of *Proteus* spp., *E. coli*, *A. aerogenes* and Paracolon bacillus.

PENICILLIN

Sensitivity Tests. The range of sensitivity to penicillin and other antibiotic agents of many species and strains of bacteria compiled from the literature and isolated from patients at Michael Reese Hospital during the past few years is shown in Tables 9.1 and 9.2. The values chosen are those required to inhibit at least two-thirds of the strains. Most of the species and strains listed in Tables 9.1 and 9.2 were recently isolated from patients who received little or no previous antibiotic therapy. Pure cultures of the isolated strains were tested for in vitro sensitivity by the tube or plate dilution methods. Results of penicillin sensitivity tests refer only to penicillin G and are given in Oxford units per cc. for the convenience of the clinician. The International Unit is defined as the specific activity contained in the International Penicillin Standard which is 0.6 micrograms of pure crystalline sodium salt of

TABLE 9.1

Range of Sensitivity of Strains of Various Gram Positive Bacteria Isolated from Patients to Antibiotics

Microorganism	Penicillin G	Streptomycin	Chlortetracycline	Chloramphenicol	Oxytetracycline	Polymyxin	Bacitracin
	Oxford units/cc.	*mcg./cc.*	*mcg./cc.*	*mcg./cc.*	*mcg./cc.*	*mcg./cc.*	*units/cc.*
Streptococcus							
Alpha hemolytic	0.01 - 2.5	3.12- 200	0.31 - 6.3	2.5 -12.5	0.25 - 1.25	>200	0.125-1.0
Beta hemolytic							
Group A	0.001- 0.08	12.5 - 100	0.08 - 3.1	1.6 - 6.3	0.08 - 0.25	200-400	0.008-0.017
Group B	0.006- 0.15	50 - 100	0.62	2.5 - 5.0	0.16 - 0.5		0.008-0.017
Group C	0.016- 0.08	6.25	0.16	2.5	0.6		0.008-0.017
Group D	2.5 ->8	12.5 - 50	0.31 - 25.0	0.62-10	0.16 - 3.0	>100	0.07 -0.15
Nonhemolytic	0.04 - 1.25						
fecalis	1.8 - 15.0	25 - 200	1.6 ->8	2.0 -25	0.125->8	>200	0.62 -5.0
Staphylococcus aureus	0.03 ->5	2.0 ->80	0.9->8	4.0 -12.5	0.125->8	>250	0.31 -1.25
Pneumococcus	0.004- 0.04	6.3 - 50	0.31 - 3.1	1.6 - 6.3	0.16 -0.25	>100	0.07 -0.31
Clostridium perfringens	0.02 - 0.1	>100	0.1	1 - 200	0.5	>1000	0.008-0.017
Cl. tetani	0.1 - 10	>100	0.1	10	0.5	>1000	

TABLE 9.2

Range of Sensitivity of Strains of Various Gram Negative Bacteria Isolated from Patients to Antibiotics

Microorganism	Penicillin G	Streptomycin	Chlortetra-cycline	Chloram-phenicol	Oxytetracycline	Polymyxin	Bacitracin
	Oxford units/cc.	*mcg./cc.*	*mcg./cc.*	*mcg./cc.*	*mcg./cc.*	*mcg./cc.*	*units/cc.*
Gonococcus	0.004– 0.028	1.6 – 6.3	0.2 – 1.6	0.4 – 3.1	0.8 – 3.1	12.5 –800	0.07 – 0.31
Meningococcus	0.016– 1.25	1.25	0.2 – 5.0	0.25	0.2 – 3.1	0.0625	
Escherichia coli	40 –200	5.0 –>80	2.0 – 25	2.0 – 25	1.25– 50	0.005– 4	170 –340
Aerobacter aerogenes	>1000	1.25–>80	2.0 – 50	4.0 – 50	1.25– 8	1.0 – 3.1	170 –340
Pseudomonas aeruginosa	>1000	5.0 – 200	50 –400	100 –400	4.0 –>8	0.1 – 4	>340
Proteus spp.*	20 – 80	2.5 – 200	6.25 –400	2.5 – 50	10 –100	>200	>340
Klebsiella pneumoniae	10 –100	0.8 – 6.3	0.2 – 25	1.6 – 5	0.62– 1.25	0.06 – 1.3	85 –170
Hemophilus influenzae	0.1 – 5.0	0.3 – 10	0.125– 4.0	0.25– 1.0	0.25– 5.0	0.006– 2.0	25.5 –102
H. pertussis	0.5 – 2.5	0.8 – 80	0.16 – 12.5	0.16– 8.0	0.2 – 12.5	0.08 – 1.0	0.625– 6.8
Shigella	40 –200	2.5 – 100	0.2 – 5.0	1 – 12.5	0.25– 12.5	0.08 – 1.6	192 –384
Salmonella							
S. typhosa	5.0 – 20	10 – 200	12.5 – 25	0.75– 12.5	2.0 – 6.0	0.06 – 1.6	192 –384
Others	20 – 50	10 – 200	6.0 – 50	2.0 – 12.5	1.6 – 4.0	0.1 – 3.1	384 –768
Paracolon		2.5 – 10	2.0	8.0	1.0 – 6.0	0.005– 0.8	

* Includes *P. mirabilis*, *P. morganii* and *P. vulgaris*.

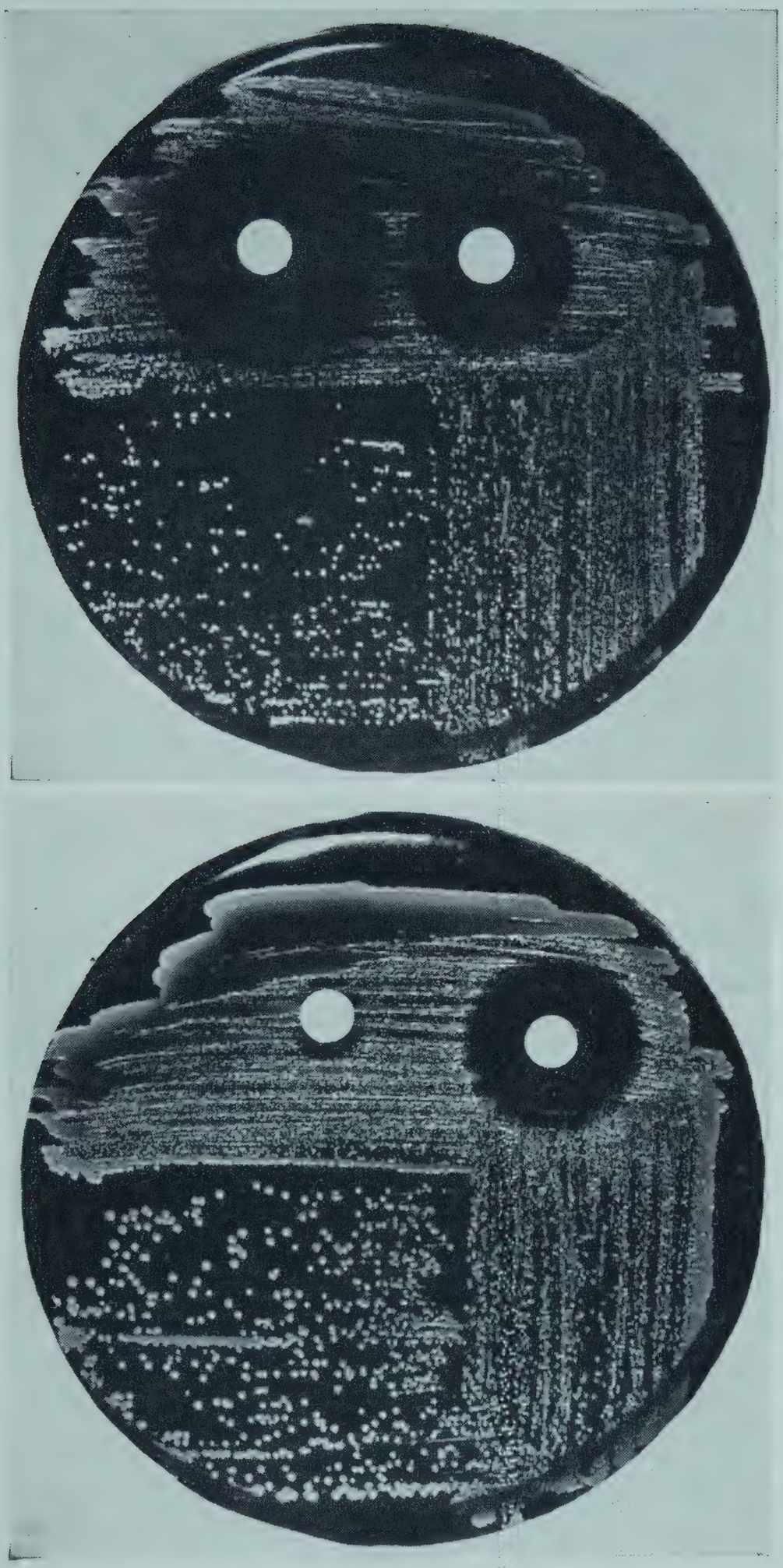


FIG. 9.1. Filter paper disc method for determining sensitivity to penicillin and streptomycin. A: a strain of *Staphylococcus aureus* very susceptible to both penicillin (left disc) and to streptomycin (right disc). B: another strain of *Staph. aureus* resistant to penicillin (left disc) but very sensitive to streptomycin (right disc). From Bondi *et al.*, 1947. Reproduced through the courtesy of the authors.

penicillin G. This unit is approximately equivalent to the Oxford unit The filter paper disc method is recommended by Lind (1953) as a simple routine screening method for determining qualitative susceptibility to penicillin and other antibiotics. The serial tube dilution method is recommended when more precise results are required as in penicillin therapy of chronic infections such as subacute bacterial endocarditis due to alpha hemolytic streptococci, nonhemolytic streptococci, etc.

A. *Filter Paper Disc Method.*

1. For details of technic and interpretation consult Bondi *et al.* (1947), Kolmer *et al.* (1951) or manufacturer's literature—Difco dehydrated sensitivity paper discs, Desi-Discs, and Commercial Solvents Dia-Disc tablets.
2. Arbitrarily, zones of growth inhibition more than 20 mm. in diameter indicate high sensitivity, 10 to 20 mm. moderate sensitivity and less than 10 mm. resistant organisms. These results are based on a filter paper disc dipped in solution of penicillin containing 50 units per cc. I have already emphasized that the filter disc method is less accurate than the tube or plate dilution technics.

B. *Serial Tube Dilution Method* (Kolmer *et al.*, 1951)

1. *Sensitive.* Microorganisms whose growth is completely inhibited by less than 0.2 Oxford units per ml. Includes all or most strains grouped in this category in Table 9.3.
2. *Moderately Sensitive.* Those microorganisms whose growth is completely inhibited by 0.2 to 2 Oxford units per ml. Some strains in this category may be resistant.
3. *Resistant.* Those requiring more than 2 Oxford units per ml. to inhibit growth. A few strains in this category such as *Streptococcus fecalis* will respond to treatment with large doses of penicillin. The author's laboratory routinely employs strain 209P *Staphylococcus aureus* to check the potency of the penicillin stock solution and technic of the test. Strain 209P *Staphylococcus aureus* may be obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington, D. C. In our hands, the penicillin sensitivity of this strain is 0.04 Oxford units per ml., while the streptomycin sensitivity reading varies from 0.625 to 1.25 micrograms per ml.

Blood Serum Levels. The amount of penicillin may be determined in blood serum or plasma, cerebrospinal fluid, transudates, exudates and urine, and reported in terms of either International Units or Oxford

TABLE 9.3

Sensitivity of Various Pathogenic Microorganisms to Penicillin

Sensitive (less than 0.2 units per cc.)	Moderately Sensitive (0.2 to 2 units per cc.)	Resistant (more than 2 units per cc.)
Alpha hemolytic streptococcus	*Actinomyces bovis*	*Aerobacter aerogenes*
Bacillus anthracis	Alpha hemolytic streptococcus (most strains)	Beta hemolytic streptococcus (Group D)
Beta hemolytic streptococcus (Groups A, B & C)	Anaerobic streptococci	*Blastomyces dermatitidis*
Borrelia novyi (relapsing fever)	*Cl. botulinum*	*Brucella* spp.
B. recurrentis (relapsing fever)	*Corynebacterium diphtheriae*	*Candida albicans*
B. vincenti	*Hemophilus ducreyi*	*Coccidioides immitis*
Clostridium perfringens	*H. influenzae*	*Donovania granulomatis* (granuloma inguinale)
Cl. septicum	*H. pertussis*	*Escherichia coli*
Cl. tetani	Nonhemolytic streptococcus (most strains)	*Histoplasma capsulatum*
Erysipelothrix rhusiopathiae (swine erysipelas)	Ornithosis virus	Influenza virus
Gonococcus	Psittacosis virus	*Klebsiella pneumoniae* (Friedlander's bacillus)
Leptospira icterohemorrhagiae (Weil's disease)	*Staphylococcus aureus* (approx. 40% of strains)	*Listerella*
Meningococcus		Lymphogranuloma venereum
Nonhemolytic streptococcus		Neurotropic Viruses
Pneumococcus		*Pasteurella tularensis*
Spirillum minus (rat bite fever)		*P. pestis* (plague)
Streptobacillus moniliformis (rat bite fever)		*Plasmodium*
Treponema pallidum		*Proteus* spp.
T. pertenue (yaws)		*Pseudomonas aeruginosa* (*B. pyocyaneus*)
		Rickettsiae
		Salmonella
		S. typhosa
		Shigella
		Staph. aureus (approx. 60% of strains)
		Streptococcus fecalis
		Toxoplasma
		Vibrio comma (cholera)

units. The assay of penicillin is accomplished by comparison of the inhibitory action of the unknown on certain standard strains of bacteria, with similar inhibitory activity in a standard solution. Three technics are in common use—the Oxford cup or cylinder-plate method, the test tube method, and the capillary tube method. These are basically similar, but differ from each other in details of apparatus and strains of organisms employed (see Kolmer *et al.*, 1951 and Welch, 1948).

The assay of penicillin in the serum and other body fluids is not a routine procedure except for the laboratory control of penicillin therapy of subacute bacterial endocarditis, osteomyelitis and other chronic infections. The blood serum level serves as a guide as to whether or not an adequate dose of penicillin is being given to maintain a therapeutic or inhibiting level of activity against the infecting organism. Penicillin diffuses readily into the other body fluids except the spinal fluid where the concentration is normally very low, but in the presence of meningeal irritation appreciable levels may occur. Rammelkamp's technic (1942) is used to determine penicillin and streptomycin blood levels at Michael Reese Hospital.

1. Collect 8 to 10 ml. of blood aseptically and allow it to clot in a sterile dry tube just before giving the next injection of penicillin or streptomycin. All specimens should be placed in the refrigerator at 5°C. immediately after collection and kept there until tested. Twenty-four hours is the maximum time that specimens can be stored before assay.

Approximately 90 per cent of the concentration of penicillin in the blood is detectable in the serum. An average blood serum level of 0.03 to 0.05 Oxford units per ml. is generally adequate for therapy of organisms that are sensitive to penicillin. This level is readily achieved for a minimum of 24 hours by the administration of 300,000 units of procaine penicillin G in oil intramuscularly as recommended by the various manufacturers. As a rule, a serum level of 2 to 3 times the in vitro sensitivity is optimal for therapy; in chronic infections such as subacute bacterial endocarditis or osteomyelitis levels 4 to 8 times the sensitivy are recommended.

The assay of blood serum or other body fluid levels is not routine in clinical practice except in the management of subacute bacterial endocarditis, chronic osteomyelitis and related infections. According to Dowling (1948) the penicillin serum concentration will rise approximately 0.1 unit per ml. for each 100,000 units given over a 24-hour period. He recommends 24-hour doses of 500,000 to 1 million units of crystalline penicillin given by continuous intramuscular or intravenous infusion for moderately severe infections; doses up to 40 million units or more have been given for more severely ill patients or infections due to relatively resistant organisms. The use of caronamide or other kidney tubule blocking agents will delay the excretion and increase the penicillin serum level. The reader is referred to Dowling in Welch (1954) for an excellent discussion of penicillin administration in clinical practice.

Direct Penicillin Assay Test. An unsatisfactory therapeutic response

s sometimes obtained when results from assaying penicillin blood levels vith an unrelated species or strain of organism are used as a guide for penicillin dosage. This is especially true when relatively resistant trains of bacteria are encountered. To meet this deficiency, we (Schlichter *et al.*, 1949) introduced the technic of assaying the bacteriostatic activity of a patient's serum level during penicillin therapy using he *isolated* etiologic organism. This technic was initially developed in he course of studies of subacute bacterial endocarditis due to resistant trains of alpha hemolytic streptococci. The success obtained in our nands has led to use of this technic as a guide to effective therapy in septicemias secondary to a variety of acute and chronic suppurative conditions with satisfactory results. Our publication should be consulted for details (Schlichter *et al.*, 1949).

STREPTOMYCIN

The testing of in vitro sensitivity of bacteria to streptomycin is an important laboratory determination because of the relative ease with which maximum resistance develops. For this reason, the decision whether a patient will receive streptomycin is often based on the results of a sensitivity determination. Tables 9.1 and 9.2 outline the range of sensitivity of various species and strains of bacteria to streptomycin. As for penicillin, the values given are those required to inhibit at least two-thirds of the strains. Lenert *et al.* (1947) point out that the sensitivity of an organism to streptomycin is influenced by age of culture, concentration of organisms, growth phase of culture, and ingredients of medium used. These factors must be kept constant in order to obtain reproducible sensitivity readings. There is no evidence that the bacterial spectrum of dihydrostreptomycin and streptomycin differs significantly. Certain Gram negative organisms, however, including *Aerobacter aerogenes*, several *Salmonella* species and *Shigella sonnei* are more sensitive to streptomycin (Waksman, 1949). *Streptomyces griseus* is the source of streptomycin, and dihydrostreptomycin is prepared by the hydrogenation of streptomycin.

Sensitivity Tests. The results of in vitro streptomycin sensitivity and assay tests are expressed in micrograms per cc. The filter paper disc method of Bondi and the serial tube dilution method as recommended for penicillin sensitivity are applicable to both streptomycin and dihydrostreptomycin, except the tubercle bacillus sensitivity test. Dilute stock solutions of streptomycin are much more stable than penicillin and may be stored for several months in the refrigerator without appreciable loss

of potency. We have found strain 209P *Staph. aureus* convenient for checking the potency of streptomycin stock solutions as well as penicillin. The Food and Drug Administration employs *Bacillus subtilus* (American Type Culture Collection 6633) as the test organism in one of the official tests for the certification of streptomycin (Welch, 1948). Other methods use *Klebsiella pneumoniae* (ATCC-9947), *B. circulans*, etc.

Filter Paper Disc Technic. High sensitivity is indicated by zones of growth inhibition greater than 15 mm. in diameter; 10–15 mm., moderate sensitivity and less than 10 mm. resistant microorganisms. As in the interpretation of penicillin sensitivity tests by this method it must be kept in mind that these figures are not absolute.

Tube or Plate Dilution Technics (see Table 9.4).

1. *Sensitive.* Microorganisms whose growth is completely inhibited by less than 10 micrograms of streptomycin per ml. Includes most strains of the species grouped in this category in Table 9.4. According to Steenken, *et al.* (1949) the majority of strains of human

TABLE 9.4

Sensitivity of Various Pathogenic Microorganisms to Streptomycin or Dihydrostreptomycin

Sensitive (less than 10 mcg. per cc.)	Moderately Sensitive (10 to 20 mcg. per cc.)	Resistant (more than 20 mcg. per cc.)
B. anthracis	*A. aerogenes* (most strains)	*A. aerogenes*
E. coli	Alpha hemolytic streptococci	*Bacteroides*
Gonococcus	Beta hemolytic streptococci (Group A & C)	Beta hemolytic streptococci (most strains)
Kl. pneumoniae	*Brucella* spp.	*Candida albicans*
Meningococcus	*C. diphtheriae*	*Cl. septicum*
Mycobacterium tuberculosis	*E. coli* (most strains)	*Cl. tetani*
Paracolon	*H. influenzae*	*Cl. perfringens*
Pasteurella pestis	*H. pertussis*	*Coccidioides immitis*
P. tularensis	Nonhemolytic streptococci (most strains)	*E. coli*
	Paracolon	*Histoplasma capsulatum*
	Pneumococcus	Nonhemolytic streptococci (most strains)
	Proteus spp.	*Pseudomonas aeruginosa* (most strains)
	Pseudomonas aeruginosa	*Salmonella*
	Salmonella spp.	*S. typhosa*
	S. typhosa	*Staph. aureus* (most strains)
	Shigella	*Strep. fecalis*
	Staph. aureus	Viruses

type tubercle bacillus are sensitive to 1 microgram of streptomycin per cc. or less. Strains highly sensitive to streptomycin in vitro are not necessarily equally susceptible in vivo. This is well illustrated by infections due to sensitive strains of *Salmonella* and *Shigella* which do not respond readily to treatment. However, strains highly sensitive in vivo usually have the same sensitivity in vitro.

2. *Moderately Sensitive.* Growth is inhibited by 10 to 20 micrograms per ml. Many strains of species in this category may be resistant.
3. *Resistant.* Strains requiring 20 micrograms per ml. or more to inhibit growth. Very few strains in this category causing systemic infections will respond to treatment even with larger dosages of streptomycin. We have found, however, that intestinal infections due to *Proteus* spp., *Ps. aeruginosa* or paracolon organisms sensitive to 80 micrograms per ml. often respond to 2 grams of oral streptomycin given daily in four equally divided doses.

An average blood serum level of 10 to 12 micrograms per ml. is usually adequate for treatment of most streptomycin sensitive microorganisms which respond in vivo. A parenteral dose of 0.5 gram of streptomycin four times daily will often maintain this blood serum level because this antibiotic is not excreted so rapidly as penicillin. A serum level at least 2 to 3 times the in vitro sensitivity is optimal for treatment except for nonhemolytic streptococci and *Staph. aureus* which may require 4 to 8 times as much in the presence of serum. Although routine assay of streptomycin in serum or other body fluids is rarely indicated, it is imperative that the proper dose of streptomycin be given for an adequate length of time on account of the relative ease with which *maximum* resistance is developed to this antibiotic.

Tubercle Bacillus Sensitivity Test. Liquid or solid media suitable for the growth of the tubercle bacillus may be used to test in vitro sensitivity to streptomycin. The digested and concentrated specimen from the patient or isolated culture is inoculated in measured amounts on tubes or bottles of nutrient agar egg yolk medium containing varying concentrations of streptomycin (Youmans *et al.*, 1950) Final concentrations of 1, 10, and 100 micrograms per ml. of streptomycin are convenient for routine tests. A control tube containing no streptomycin must be included. By this method, results of highly positive specimens may be evident in 2 to 4 weeks.

Cummings *et al.* (1949) have described a slide culture technic for rapid streptomycin sensitivity testing. The method is easily performed and gives sensitivity readings which compare favorably with the more standard methods employing inspissated egg media or egg yolk agar.

Blood Serum Levels. The Rammelkamp technic used for assay o penicillin serum and other body fluid levels is recommended for strepto mycin assay. Chemical methods for streptomycin assay are describe by Welch (1948). Streptomycin is distributed evenly in the extracellula body fluid following parenteral inoculation, but practically none normall appears in the spinal fluid. After oral administration, streptomycin i poorly absorbed from the gastrointestinal tract and most of it is elimi nated in the feces.

ISONIAZID

Isoniazid (isonicotinic acid hydrazide) is bacteriostatic and bactericida against the tubercle bacillus both in vitro and in vivo. This substance has a very narrow antibacterial spectrum, being ineffective in vitro against the common Gram positive and Gram negative pathogenic bacteria. It is also inactive against Rickettsia and viruses, but may possess slight fungicidal activity. It is of interest that isoniazid is active against both intracellular as well as extracellular tubercle bacilli in vivo. Resistance develops readily in vitro, and Hobby *et al.* (1953) have demonstrated a synergistic action between isoniazid and streptomycin. Recently, Middlebrook *et al.* (1953) have made the significant observation that tubercle bacilli resistant to 10 micrograms or more of isoniazid per ml. may become partially or completely attentuated for the guinea pig.

Taken orally isoniazid is absorbed and excreted rather rapidly. Therapeutic serum levels (0.5 micrograms/ml.) occur within one to four hours after oral doses of 3 to 5 mg. per kg., and the drug is largely eliminated in the urine within 24 hours. The blood brain barrier is readily passed and therapeutic concentrations are found in the cerebrospinal fluid. (Elmendorf *et al.* (1952)).

Youmans nutrient agar egg yolk medium is satisfactory for in vitro sensitivity tests. The interpretation of sensitivity tests is as follows:

1. *Sensitive.* Tubercle bacilli strains whose growth is inhibited by 0.1 to 1 micrograms per ml.
2. *Resistant.* Tubercle bacilli strains requiring more than 1 microgram per ml. to inhibit growth.

CHLORTETRACYCLINE (TRADE NAME AUREOMYCIN)

Chlortetracycline is derived from a strain of *Streptomyces aureofaciens* and appears to be bacteriostatic rather than bactericidal except in high concentration. On a weight basis chlortetracycline is less effective than penicillin against most of the coccal organisms, but is approximately as

ffective as streptomycin against most of the Gram negative bacilli. 'ables 9.1 and 9.2 summarize the range of in vitro sensitivity tests of arious species of bacteria to chlortetracycline.

Sensitivity Tests. Tests for sensitivity to chlortetracycline can be arried out by the Bondi filter paper disc, serial tube dilution or streak late methods. Results are expressed in micrograms per ml. Because f the known *instability* of chlortetracycline, care must must be taken to repare solutions fresh from the crystalline state. The activity of this ntibiotic deteriorates rapidly in alkaline solution at room temperature. Ve have found a stock solution of 80 micrograms per ml. of chlortetra- ycline in distilled water convenient for use in the tube dilution test nd relatively stable for 18 to 24 hours at room temperature but not at 7°C. The stock solution may be stored for long periods if frozen and ept in dry ice (solid carbon dioxide) or at −20°C.

Technical difficulties are often encountered in sensitivity tests as well s in titrations of chlortetracycline in the blood serum because this anti- iotic deteriorates very rapidly in dilute alkaline solutions. The pres- nce of serum also diminished its inhibitory action in vitro. Finland *t al.* (1948) and Alexander *et al.* (1949b) both state that the use of solid ulture media for sensitivity tests is more reliable than the tube dilution nethod. The size of the inoculum influences the concentration of hlortetracycline required to inhibit bacterial growth in liquid media, whereas it has little effect, within a wide range of inoculum size, on the nd points obtained in solid media. Finland (1948) finds that chlor- etracycline is much more effective in an acid than in an alkaline medium —the reverse of streptomycin.

Filter Paper Disc Technic. Results are based on discs dipped in 500 icrograms /ml. aqueous solution of chlortetracycline. Zones of growth nhibition greater than 17 mm. in diameter indicate high susceptibility, 3 to 17 mm. moderate sensitivity and less than 13 mm. resistant strains. *These sensitivity ranges are very arbitrary because of the instability of hlortetracycline.*

Tube or Plate Dilution Technics. It must be kept in mind that the nterpretation of results, especially when obtained by the tube dilution nethod, may be subject to considerable error for several reasons. First, he concentration of chlortetracycline required to inhibit growth varies ith the size of the inoculum in liquid media. For this reason, the late dilution method is preferred for sensitivity tests. Secondly, the nstability of chlortetracycline at 37°C. may result in incorrect deter- inations. In general our experience indicates that strains sensitive to

less than 2 micrograms per ml. are very susceptible, 2 to 8 micrograms moderately sensitive and over 8 micrograms resistant.

Blood Serum Levels. Levels are determined by the serial tube dilution method described for assaying penicillin with the following exceptions. Bacillus ✻5 Dornbush (a strain of *B. cereus*) is used as the test organism. The culture is incubated in veal infusion broth for 6 hours at room temperature, diluted 1:10,000 with sterile saline solution, and 0.05 ml. amounts are added to each of the graded serum dilution tubes. All tubes are incubated at *room temperature* for 18 hours and read for visible growth. End points are generally clear cut, and the sensitivity of the test organism usually varies from 0.016 to 0.032 micrograms per ml.

The assay of blood serum or other body fluids is also only a rough approximation for the above mentioned reasons. Most authors regard an average chlortetracycline serum level of 2 to 4 micrograms per ml. as a therapeutic concentration. This level can usually be obtained by the administration of an oral dose of 1 gram stat. and 0.5 gram every 6 hours. Intravenous doses of 50 to 100 micrograms rapidly produce serum concentrations between 2.5 to 10 micrograms per ml., but the level gradually declines to 0.15 micrograms per ml. during the next 6 to 8 hours (Brainerd *et al.*, 1949).

Chlortetracycline may be administered orally, subcutaneously or intramuscularly and is excreted in the urine. It is rapidly absorbed from the gastrointestinal tract and is probably distributed in both the extracellular and intracellular tissues (Long *et al.*, 1950). A large part of the oral dose is apparently destroyed by the intestinal contents. Some chlortetracycline diffuses into the spinal fluid normally but in lower concentrations than it occurs in the blood serum.

CHLORAMPHENICOL (TRADE NAME CHLOROMYCETIN)

Chloramphenicol is a product of *Streptomyces venezulae*. Crystalline chloramphenicol can now be produced synthetically and is a nitrobenzene compound in chemical structure. Chloramphenicol is a very stable antibiotic in contrast to chlortetracycline. It is unaffected by boiling for several hours and is stable over the pH range from 2 to 9. It is relatively insoluble. Aqueous solutions containing 0.25 per cent concentration may be stored for several months in the refrigerator at 5°C., but solutions containing less than 1.0 mg. per ml. deteriorate within a few days or weeks of storage (Smadel, 1949). Chloramphenicol exerts a bacteriostatic action in vitro, but is bactericidal at higher concentra-

tions against a wide range of bacteria (Schoenbach, 1949). It is most effective against Gram negative bacilli (Tables 9.1 and 9.2). Results are expressed in micrograms per ml.

Sensitivity Tests. The Bondi filter paper disc and the serial tube dilution methods as described for penicillin are suitable for sensitivity tests. A convenient stock solution for use in the serial tube dilution method consists of 80 micrograms per ml. in sterile distilled water.

Filter Paper Disc Technic. High sensitivity is indicated by zones of growth inhibition greater than 20 mm. in diameter, moderate sensitivity by zones 10 to 20 mm. in diameter and resistant strains by zones less than 10 mm. in diameter.

Tube or Plate Dilution Technics. The in vitro sensitivity test to chloramphenicol is not subject to the same limitations as chlortetracycline. This antibiotic is relatively stable, and reproducible results are readily obtained by either the tube or plate dilution methods. We consider strains sensitive to less than 2 micrograms per ml. very susceptible, 2 to 6 micrograms moderately sensitive and over 6 micrograms resistant.

Blood Serum Levels. Both chemical and biological tests are available for chloramphenicol assay. The Rammelkamp technic described for penicillin assay is suitable. *Shigella paradysenteriae* (Sonne) Parke, Davis № 04628 is used as the test organism. Smadel (1949) recommends a modification of the turbidimetric method of Smith *et al.* (1948) which has been used extensively at the Army Medical Department Research and Graduate School. In this technic the amount of crystalline drug in a known standard solution which is required to produce 50 per cent inhibition of growth of *Shigella sonnei* is determined spectrophotometrically. This is compared with the inhibiting capacity of varying dilutions of the unknown material. Smadel states that this method is satisfactory for estimating the drug levels in serum, urine and spinal fluid and, with some slight modifications, milk and bile. The chemical method (Glasko *et al.*, 1949) compares favorably with the bioassay for blood and spinal fluid levels. The presence of sulfonamides, however, interferes with the chemical test.

Chloramphenical can be detected in the blood serum within 30 minutes after an oral dose of 1 gram; the concentration reaches a peak of 5 to 10 micrograms per ml. in about 2 hours and then falls steadily to undetectable levels after 8 hours (Smadel *et al.*, 1949). Consequently, doses of 0.25 to 0.5 grams are given at intervals ranging from 4 to 6 hours to maintain the serum level. Administration of a 2 gram oral dose also produces a high serum level within 30 minutes which is still 5 micrograms per ml. or

more at 8 hours, but is no longer detectable at 24 hours. For this reason sustaining doses of 0.5 to 1 gram are given at 6 to 8 hour intervals. An average blood level of 5 micrograms of chloramphenicol per ml. is considered a therapeutic level for most susceptible organisms. Smadel states that the amount of chloramphenicol in the spinal fluid, bile and milk is approximately half that found simultaneously in the blood.

Chloramphenicol is readily absorbed from the gastrointestinal tract as demonstrated by the rapid appearance of relatively high concentrations in the body fluids and passage through the placental and blood-brain barriers. It is excreted chiefly in the urine, and appears in appreciable quantities within 30 minutes after a single oral dose (Long *et al.*, 1950).

OXYTETRACYCLINE (TRADE NAME TERRAMYCIN)

Oxytetracycline is an antibiotic described by Finlay *et al.* in 1950. This substance is derived from *Streptomyces rimosus* and is relatively nontoxic. Oxytetracycline is sparingly soluble in water at pH 7, but can be readily dissolved at pH 8.3 to 8.5 by the addition of NaOH to form the sodium salt or by lowering the pH to 2 by the addition of HCl (Linsell *et al.*, 1950). Crystalline oxytetracycline is soluble in water to the extent of 0.25 mg. per ml. at 25°C. In contrast to chlortetracycline, oxytetracycline is stable over long periods at room temperature in aqueous solution at about pH 2 to 5 (Finlay *et al.*, 1950). Unlike chlortetracycline, it also appears to be fairly stable in the presence of serum (Herrell *et al.*, 1950). The action of oxytetracycline, like chlortetracycline and chloramphenicol, is predominantly bacteriostatic, and higher concentrations acting on smaller numbers of bacteria are also bactericidal. The antibacterial spectrum of oxytetracycline is remarkably similar to that of its analogue chlortetracycline (Tables 9.1 and 9.2). The unit is defined as one microgram of pure anhydrous amphoteric compound.

Sensitivity Tests and Assay. The standard methods for assay of other antibiotics can be used with equal success for oxytetracycline. We have found the technics for sensitivity tests and assay described for chlortetracycline equally applicable to oxytetracycline, except the tubes are incubated at 37°C. instead of at room temperature. A stock solution consisting of 80 micrograms per cc. in sterile distilled water is suitable and may be stored in the refrigerator for approximately three weeks before discarding. The standard assay test organism is *Klebsiella pneumoniae* P.C.I. 602, but we find *Staph. aureus* 209P satisfactory. The latter has a sensitivity of approximately 0.5 micrograms per ml. in our test.

Filter Paper Disc Technic. Interpretation same as chlortetracycline.

Tube or Plate Dilution Technics. Interpretation same as chlortetracycline.

Blood Serum Levels. Individuals who receive 1 to 1.25 grams of oxytetracycline by the oral route every 6 hours will usually maintain a serum level of 4 to 8 micrograms per ml. throughout the 24-hour period (Herrell et al., 1950). An average level of 4 to 8 micrograms per ml. is ample for treatment of most oxytetracycline sensitive organisms. According to Sayer *et al.* (1951), levels of 5 to 10 micrograms per ml. are present one hour after administration of 250 mg. of the intravenous preparation, and there is a progressive decrease to levels of 1 to 5 micrograms per ml. after 12 hours.

Long *et al.* (1950) report that oxytetracycline passes through the placenta and can be found in fetal blood. It also passes into the bile and milk. Only small amounts are detected in the spinal fluid of normal individuals. Twenty-five per cent or more may be recovered in the urine during the 24-hour period following the inoculation of a single therapeutic dose of oxytetracycline.

TETRACYCLINE

The tetracyclines are broad spectrum antibiotics which include the recently developed tetracycline (Trade names Achromycin, Tetracyn and Polycycline) and its two analogues chlortetracycline and oxytetracycline. Tetracycline is a crystalline antibiotic which is prepared either by the catalytic hydrogenation of chlortetracycline or by fermentation of a *Streptomyces sp.* (Boothe *et al.*, 1953). Structurally it differs from chlortetracycline by the absence of a chlorine atom at carbon seven and from oxytetracycline by the absence of an OH group at carbon five. The three antibiotics have similar patterns of antibacterial activity, but differ somewhat in chemical stability (Welch, 1954). Tetracycline is similar to oxytetracycline in stability but is superior to chlortetracycline in this respect. The three isomers can be differentiated by paper chromatographic analysis. Thus far the use of tetracycline is accompanied by a lower incidence of gastrointestinal symptoms than is the use of comparable amounts of chlortetracycline or oxytetracycline (Finland *et al.*, 1954).

The tetracycline antibiotics are highly effective against a wide variety of both Gram positive and Gram negative bacteria (Welch, 1954). They are also very effective against all rickettsial infections and the lymphogranuloma venerum group (Perlman *et al.*, 1954). The sensitivity of various species of bacteria to the three antibiotics is quite

similar—this is true for both highly sensitive and resistant cultures. There is essentially complete cross resistance between the three analogues (Finland *et al.*, 1954). Welch *et al.* (1954), however, have recently reported chlortetracycline to be considerably more active against certain strains of staphylococci than the other two tetracycline drugs. The tetracyclines are primarily bacteriostatic agents but may show a bactericidal effect in high concentration (English *et al.*, 1953). In vitro resistance is gained slowly in a gradual, stepwise pattern comparable to that of penicillin. Tetracycline is absorbed from the gastrointestinal tract following oral administration and subsequently appears in the various body fluids, including blood serum, cerebrospinal fluid, abdominal fluid and urine. Following intravenous administration, tetracycline produces higher serum and cerebrospinal fluid concentrations than comparable dosages of oxytetracycline or chlortetracycline (Wood *et al.*, 1953).

ERYTHROMYCIN AND CARBOMYCIN

Erythromycin (trade name Ilotycin or Erythrocin) is produced by *Streptomyces erythreus* (McGuire *et al.* 1952), and carbomycin (trade name Magnamycin) is produced by *S. halstedii* (Tanner *et al.* 1952). Both erythromycin and carbomycin resemble penicillin in affecting Gram positive bacterial infections but differ in also being active against certain large viruses and rickettsiae. Erythromycin, however, is considerably more active than carbomycin. Their antibacterial activity against the Gram negative bacteria is low except for the gonococcus and *H. influenzae* (Welch *et al.* 1952). Erythromycin appears to be less effective than penicillin against most Gram positive bacterial and gonococcal infections. Its chief use has been in the therapy of infections due to penicillin-resistant strains of staphylococci.

Pharmacologically, erythromycin and carbomycin differ markedly. Therapeutic concentrations of erythromycin occur in the blood and urine following oral administration (Kirby *et al.* 1953), while carbomycin given by the same route is not readily demonstrated in the blood except in jaundiced patients. Heilman *et al.* (1952) have reported that no cross resistance apparently exists between erythromycin and chlortetracycline, chloramphenicol or oxytetracycline. These authors also failed to find any naturally occurring strains of staphylococci resistant to erythromycin. Cross resistance between carbomycin and erythromycin has been demonstrated by Finland *et al.* (1952). It was further reported that on a weight basis erythromycin is 4 to 16 times more bactericidal

than carbomycin, and that high degrees of resistance to both of these antibiotics can be developed readily *in vitro*. For therapy Heilman *et al.* (1952) recommend an oral dose of erythromycin of 0.4 to 0.5 gram every six hours. Multiple doses of 0.5-gram coated tablets gave blood concentrations ranging from 1 to 8 micrograms per cc. four and six hours after the last dose, while blood levels ranged from 0.5 to 4 micrograms per cc. 9 hours after the last dose. It was also noted that in the absence of inflammation erythromycin does not readily traverse the blood-brain barrier and only traces of the drug can be detected in the cerebrospinal fluid of normal subjects. The use of carbomycin according to Finland *et al.* (1953a) in the therapy of susceptible bacterial infections has given inferior results to the use of erythromycin in similar cases.

Sensitivity Tests. Tests for sensitivity to erythromycin can be carried out by the Bondi filter paper disc, serial tube dilution or plate dilution technics. Erythromycin Difco discs are available in 3 concentrations, 0.5, 1 and 10 micrograms.

Tube or Plate Dilution Technic

1. *Sensitive.* Microorganisms whose growth is completely inhibited by less than 1 microgram of erythromycin per ml.
2. *Moderately Sensitive.* Growth is completely inhibited by 1 to 4 micrograms per ml.
3. *Resistant.* Strains requiring more than 4 micrograms per ml. to inhibit growth.

BACITRACIN

Bacitracin is an antibiotic produced by the Tracey I strain of *B. subtilis* (Johnson *et al.*, 1945). It is water soluble and stable in acid solutions but unstable in alkaline solutions with a pH greater than 9. It is thermostable and may be boiled for 15 minutes without destruction. Bacitracin has a range of antibacterial activity similar to that of penicillin. Meleney *et al.* (1948) state that bacitracin has proved effective against most strains of hemolytic and nonhemolytic streptococci, coagulase positive staphylococci, pneumococci, gonococci and all anaerobic cocci in general. It is also effective against the gas gangrene organisms, the tetanus bacillus, the diphtheria bacillus, the spirochaete of syphilis, actinomyces and the protozoan *Endamoeba histolytica*. Tables 9.1 and 9.2 give the range of sensitivity of various species of bacteria to bacitracin. At present bacitracin is recommended for topical application only because of its inherent toxicity.

Sensitivity Test and Assay. Procedures for sensitivity test and assay

of bacitracin are described by Welch (1948). We have found the tube dilution method for penicillin suitable. A stock solution containing 80 units per ml. in sterile distilled water may be used for 1 month if stored in the refrigerator. *Micrococcus flavus* is the standard test organism. The unit of bacitracin is tentatively defined as "the amount which, when diluted 1:1,024 in a series of twofold dilutions in 2 ml. of beef infusion broth, completely inhibits the growth of a stock strain (Chanin) of Group A hemolytic streptococcus, when the inoculum used to seed the tubes is 0.1 ml. of a 10^{-2} dilution of an overnight culture in blood broth." The antibiotic activity of bacitracin is not impaired by the presence of blood, serum or pus.

Filter Paper Disc Technic. Bacitracin filter paper discs are prepared by dipping them in a solution containing 75 units per ml. (Spaulding, 1950). A zone of growth inhibition greater than 20 mm. indicates high sensitivity, 10 to 20 mm. moderate sensitivity and less than 10 mm. resistant strains.

Tube or Plate Dilution Technics. Strains sensitive to less than 1 unit of bacitracin per ml. are very susceptible, 1 to 5 units moderately sensitive and over 5 units resistant.

Blood Serum Level. This procedure is not carried out routinely in the hospital laboratory because the parenteral use of bacitracin is contraindicated with present preparations. Those who are interested in the absorption, distribution, excretion and toxicity of bacitracin in man are referred to Welch (1954) and Zintel et al. (1949).

POLYMYXIN

Polymyxin is an antibiotic obtained from filtrates of *Bacillus polymyxa* or *B. aerosporus.* The following types obtained from strains of *B. polymyxa* have been described: polymyxin A (aerosporin or bacillosporin A) B, C and D (Schoenbach, 1949; Kagan *et al.*, 1951). Each of these types are polypeptides but differ from the others in amino acid composition. Polymyxin E, the most recent preparation, is identical with polymyxin A in qualitative composition but differs in having a speed of partition chromatography resembling that of polymyxin B. Polymyxin is most effective in an acid pH. It is quite heat stable and is not inhibited by the presence of serum or pus.

The polymyxins are bactericidal rather than bacteriostatic and are unique in that their activity is *restricted* to Gram negative bacteria. Gram positive bacteria are completely resistant. The in vitro activity of polymyxin B against various species of bacteria is shown in Tables

9.1 and 9.2; the action of polymyxin D is essentially the same. Weight for weight polymyxin is considerably more effective in vitro than streptomycin against several of the Gram negative bacilli (Kagan *et al.*, 1951). The polymyxins are too toxic for general use but are of value in certain infections resistant to the other antibacterial agents (see section on clinical use of sulfa drugs and antibiotics).

Sensitivity Test. The standard of reference is a polymyxin A hydrochloride of high chemical purity, containing 10,000 bioassay units of antibiotic activity per milligram of dry substance. Because of its lower toxicity for clinical use (Kagan *et al.*, 1951), this laboratory employs polymyxin B for sensitivity tests and bioassay. Polymyxin B hydrochloride (Burroughs Wellcome & Co., Tuckahoe 7, N. Y.) is dissolved in sterile distilled water and diluted to a concentration of 80 micrograms per ml. The stock solution may be kept in the refrigerator for at least 2 weeks without significant loss of potency. The in vitro tube dilution sensitivity test described for penicillin is used for polymyxin. The tubes are incubated for 18 hours, and the sensitivity is considered the least amount of drug necessary for inhibition of growth.

Tube or Plate Dilution Technics. Sensitivity tests for this antibiotic are not done routinely except in serious infections due to strains of *Ps. aeruginosa* or other Gram negative bacilli which are resistant to the less toxic antibacterial agents. Organisms with a sensitivity of less than 1.0 microgram per ml. of polymyxin usually respond readily to therapeutic doses of polymyxin B.

Serum Level Assay. The method used for determination of serum polymyxin concentrations is the serial tube dilution technic described for penicillin assay. The standard test organism is the McCleod strain of *E. coli* which has a polymyxin sensitivity of 0.3 micrograms per ml. Polymyxin given orally is not absorbed by the gastrointestinal tract to any significant degree (Kagan *et al.*, 1951). Schoenbach (1949) found that no polymyxin was detected in the spinal fluid after intramuscular administration, even in the presence of a purulent meningitis. He also reported a lag in urinary excretion for approximately 12 hours after intramuscular injection. The drug then appeared in the urine in increasing amounts, so that approximately 60 per cent was excreted 72 to 96 hours after administration.

Intramuscular injection of 20 mg. of polymyxin B every 4 hours is generally adequate to maintain a therapeutic serum level of 2 to 5 micrograms per ml. in normal individuals. A single injection of 40 mg. will produce significantly higher levels, but the patients are more apt to

have toxic effects. For further details of polymyxin blood levels, dosage and toxicity see publications by Schoenbach (1949), Jawetz *et al.* (1949) and Kagan *et al.* (1951).

NEOMYCIN

Neomycin is an antibiotic isolated from *Streptomyces fradiae* (Waksman *et al.*, 1949). It is reported to be effective against a wide variety of Gram positive and Gram negative bacteria except anaerobic Clostridia and Bacteroides (Schweinburg *et al.*, 1952; Dearing *et al.*, 1953). This antibiotic is water soluble, basic and relatively heat stable. It is bactericidal and consists of at least three closely related chemical substances designated as neomycin A, B and C. Neomycin B which makes up over 90 per cent of commercial neomycin has a potency of 86 units per mg. against *B. subtilis* and 265 units per mg. against *K. pneumoniae* (Welch, 1954).

The nephrotoxic and ototoxic effects of neomycin preclude its parenteral use for the present. Orally, it is poorly absorbed from the intestinal tract and is largely excreted in the feces. Poth *et al.* (1950) believe that neomycin is an excellent intestinal antiseptic. They recommend a daily oral dose of 0.1 gram per kg. of body weight divided into six portions and given on a 4-hour schedule. Neomycin is also recommended for topical use in the treatment of pyogenic skin infections.

The serial tube dilution method described for penicillin is satisfactory for neomycin sensitivity tests and assay of body fluids (Waksman *et al.*, 1949). Spaulding *et al.* (1951) recommend that discs be dipped in an aqueous solution containing 300 units of neomycin per ml. for the Bondi disc-testing method. Difco discs are available in 3 concentrations—10, 30 and 60 micrograms.

INTERPRETATION OF SENSITIVITY TESTS

For the most part there is some correlation between the results of in vitro sensitivity tests and therapeutic response to the antibiotic agents, providing the proper dosage is given for a sufficient length of time. Moreover the sensitivity test must be carried out by a valid technic applicable to both a given antibiotic and the species of bacteria being tested. Otherwise the conditions of the test may profoundly influence the results, and a false reading will be obtained. Sensitivity tests of certain of the Gram negative bacteria, especially the genus *Salmonella*, are not necessarily correlated with therapeutic effect. Thus many strains of *Salmonella* are inhibited in vitro by relatively small concentra-

tions of streptomycin, chlortetracycline or chloramphenicol but fail to respond in vivo although adequate levels are readily attained in the blood or other body fluids. *Salmonella typhosa* is sensitive in vitro to all of these antibiotics, but typhoid patients respond only to chloramphenicol.

Some clinicians are of the opinion that little is gained from routinely testing the sensitivity of microorganisms isolated from patients and prefer to rely on published data and previous clinical experience. Others feel that sensitivity tests should be done routinely on all strains of coagulase positive staphylococci, alpha hemolytic, nonhemolytic streptococci and *Streptococcus fecalis* isolated from cases of subacute bacterial endocarditis and other organisms which continue to be cultured from blood or other body fluids after starting antibacterial therapy. Routine sensitivity tests for *Pseudomonas aeruginosa* and *Proteus* spp. are also advisable because of the great variability in response of strains to sulfa drugs and antibiotics. We have adopted the latter procedure in our institution. The validity of such a position is emphasized by reference to Tables 9.1 and 9.2 which show the great variability of sensitivity ranges of different strains of a given species of bacteria to the antibiotic agents. The fact that different species also vary markedly in sensitivity to the same antibiotic is readily seen. In general, the sensitivity of any species or strain of organism to a given antibiotic is independent of its susceptibility to the other antibacterial agents, except the tetracycline antibiotics.

Although the in vitro sensitivity test with the exception noted above is generally a useful guide to the value of a given antibiotic for therapy, there are several other factors which have a role in determining therapeutic effectiveness. These include absorption, distribution and excretion of the antibiotic as well as its plasma-binding properties and toxicity (Frank *et al.*, 1950a). In addition the nature of the lesion, the conditions that prevail at the site of the infection and the accessibility of the antibiotic to the bacteria are important. Furthermore, the interpretation of the sensitivity test does not take into consideration the antibody response of the patient to the infection.

Many factors must be taken into consideration in order to explain the discrepancy in results in antibiotic sensitivity tests. The results of sensitivity tests currently employed can be made to vary by altering any one of the following factors (Jackson *et al.*, 1951): (1) size of the inoculum; (2) length of the incubation period; (3) choice of the endpoint as complete or partial inhibition; and (4) composition of the culture medium (Neter *et al.*, 1952). Most authorities feel that sensitivity tests

as ordinarily performed are only general qualitative guides, valuable for differentiating susceptible from nonsusceptible organisms. Spaulding *et al.* (1951) have recommended that sensitivity tests be performed in all fulminating infections and in infections not responding after 72 hours of antibiotic therapy, or in cases of relapses during therapy or following change in bacterial flora. It is a common observation that clinical results do not always parallel antibiotic laboratory tests. Jackson *et al.* (1951) believe that in vitro sensitivity tests are useful as a general qualitative guide to differentiate susceptible from nonsusceptible organisms. Even under rigidly controlled test conditions the serial dilution methods are accurate only within the range of a two fold dilution of the antibiotic, while the antibiotic filter paper-disc technic gives results that vary in triplicate as much as ±25% (Spaulding *et al.*, 1951). For all these reasons the results of sensitivity tests obviously *cannot* be interpreted in terms of absolute figures. With the above mentioned reservations in mind, clinical experience tends to confirm the validity of arbitrarily grouping the various species of microorganisms into sensitive, moderately sensitive and resistant categories.

SYNERGISM AND ANTAGONISM

Physicians sometimes employ combinations of antibiotics and the sulfonamides. This is done with the hope that the two drugs will be synergistic or at least additive. There is both laboratory and clinical evidence (Hunter, 1950) that penicillin and streptomycin are synergistic, and this combination has been used successfully in the treatment of subacute bacterial endocarditis due to the enterococcus. Herrell *et al.* (1950) have reported that oral chlortetracycline and dihydrostreptomycin intramuscularly are more effective in the treatment of brucellosis than chlortetracycline alone. There is also evidence that penicillin and bacitracin are synergistic both in vitro and in vivo (Johnson *et al.*, 1950).

Synergism indicates the ability of two antimicrobial drugs acting together to increase the bactericidal or cure rate beyond that obtainable by simple additive effects of the agents. Antagonism, on the other hand, refers to an effect produced by two antimicrobial drugs in combination that is less than that achieved by either drug alone. Jawetz *et al.* (1952a) have conducted many laboratory and clinical observations on this subject and have divided the commonly used antibiotics into two groups:

I. Penicillin, bacitracin, streptomycin, and neomycin.
II. Chlortetracycline, oxytetracycline, chloramphenicol and the newer antibiotics erythromycin and carbomycin.

Members of group I are primarily bactericidal and are frequently synergistic with each other, occasionally indifferent but generally never antagonistic. Group II antibiotics are primarily bacteriostatic and are usually neither synergistic nor antagonistic, but often exhibit simple additive effects. The sulfonamides probably belong to group II. The place of polymyxin B is still uncertain. Combinations of group I and II are very complex and depend on the relative susceptibility of the microbial strain. The group II compound often reduces the effectiveness of the group I compound if the invading organism is sensitive to the latter. On the other hand, the group II compound may be expected to act synergistically with the group I compound if the organism in question is resistant to the group I compound. This relationship does not always hold true in clinical practice and cannot be predicted with certainty. Combinations of the two groups should be avoided unless synergism has been demonstrated in vitro. If it becomes necessary to use a combination of an antibiotic from groups I and II, it is important to give doses that will result in adequate therapeutic concentrations of each antibiotic at the site of the infection (Dowling *et al.*, 1953). Jawetz *et al.* (1952b) in a report to the Council on Pharmacy and Chemistry of the American Medical Association "emphasize the impossibility of labeling certain drug pairs as 'synergistic' or 'antagonistic' and stress the desirability of laboratory guidance in those occasional difficult situations where drug combinations are essential for cure."

CLINICAL USE OF SULFA DRUGS AND ANTIBIOTICS

An adequate discussion of the clinical use of the antibacterial agents is not possible in this chapter because of the lack of space. The reader is referred to Welch (1954), and recent textbooks of clinical medicine. I feel that it is profitable to summarize briefly the present clinical indications for the use of the various agents, especially because the therapy of infections has been considerably complicated since the advent of the newer antibiotics. Tables 9.5 and 9.6 list the therapeutic agents of choice for systemic use in each of the various infectious diseases. Obviously, the determination of the drug of choice is somewhat arbitrary, but I have attempted to express the current opinions in the literature. Since new work is constantly appearing, it is realized that the data presented concerning the newer antibiotics is subject to frequent revision.

The sulfonamides (gantrisin, sulfadiazine or sulfamerazine) are among the drugs of choice in meningococcal meningitis, *Shigella* infections, *Proteus* infections, plague, glanders, lymphogranuloma venereum and chancroid. Infections due to antibiotic resistant strains of *Aerobacter*

TABLE 9.5

Antibacterial Agents of Choice for Treatment of Various Infections

Microorganism	Sulfa Drugs	Penicillin G	Streptomycin	Chlortetracycline	Chloramphenicol	Oxytetracycline	Erythromycin
Streptococcus							
Alpha hemolytic	IV	I	III	II	0	U	I
Beta hemolytic	II	I	IV	II	IV	III	II
Nonhemolytic	III	I	0	II	0	U	U
S. fecalis (Enterococcus)	0	II	III	I	II	IV	U
Staph. aureus	III	II	0	I	II	II	I
Pneumococcus	II	I	II	II	III	II	II
Meningococcus	I	I	0	II	U	II	U
Gonococcus	III	I	II	II	III	II	U
C. diphtheriae	0	I + A.T.	0	II	U	U	U
M. tuberculosis	0	0	I + P.A.S.	0	0	0	0
M. leprae	I†	0	0	U	U	U	U
B. anthracis	II	I	0	II	U	II	U
Cl. perfringens	0	I + A.T.	0	II	U	II	U
Cl. tetani	0	I + A.T.	0	U	U	U	U
E. coli	II	0	II	I	I	I	0
Kl. pneumoniae (Friedlander's bacillus)	III	0	II	I	II	II	0
Proteus spp.	I	0	II	0	III	U	0
A. aerogenes	I	0	II	I	I	I	U
Ps. aeruginosa (*B. pyocyaneus*)	II	0	II‡	II	U	II	0
Salmonella							
S. typhosa	0	0	0	0	I	0	0
Others	III§	0	II§	II§	II§	I§	0
Shigella	I	0	0	I	II	I	0
Pasteurella							
P. pestis (plague)	I	0	II	U	U	U	0
P. tularensis	0	0	I	II	II	II	0
Brucella spp.	0	0	II + S.D.	I + strep.	II	I + strep.	U
Listerella	I	0	0	I	U	U	U
Vibrio comma (cholera)	U	0	II	U	U	U	U
Hemophilus							
H. influenzae	IV	0	III + S.D.	II + S.D.	I + S.D.	III	U
H. pertussis	0	0	II	I	I	I	U
H. ducreyi	I	0	I	II	III	II	U

I–IV = Approximate order of preference. 0 = Ineffective. U = Unknown. A.T. = Antitoxin. P.A.S. = P-aminosalicylic acid. S.D. = Sulfadiazine. Strep. = Streptomycin.

† Promin or Diasone.

‡ Polymyxin B is best.

§ Only occasionally effective.

TABLE 9.6

Antibacterial Agents of Choice for Treatment of Various Infections

Microorganism	Sulfa Drugs	Penicillin G	Streptomycin	Chlortetracycline	Chloramphenicol	Oxytetracycline	Erythromycin
Spirochetes							
Treponema pallidum	0	I	0	II	II	II	U
T. pertenue (yaws)	0	I	0	U	U	U	U
B. recurrentis	0	I	0	I	I	U	U
B. vincenti	0	I	0	U	U	U	U
Leptospira icterhemorrhagiae (Weil's disease)	0	I	0	I	U	U	U
Donovani granulomatis (granuloma inguinale)	III	0	II	I	I	II	U
Streptobacillus moniliformis (rat bite fever)	0	I	I	I	U	U	U
Bacteroides	II	0	II	I	U	II	U
Malleomyces mallei (glanders)	I	0	0	U	U	U	U
Bartonella	0	0	0	U	I	U	U
Rickettsia							
R. prowazeki	0	0	0	I	I	I	U
R. mooseri	0	0	0	I	I	I	U
R. conori	0	0	0	I	I	I	U
R. orientalis	0	0	0	I	I	I	U
R. akari (rickettsialpox)	0	0	0	I	I	I	U
Coxiella burnetii (Q fever)	0	0	0	I	I	I	U
Viral agents							
Psittacosis	II	II + S.D.	0	I	I	I	U
Lymphogranuloma venereum	I	0	0	I	I	I	U
Primary atypical pneumonia	0	0	0	I	I	I	U
Herpes simplex	0	0	0	0	0	0	0
Herpes zoster	0	0	0	0	0	0	0
Chickenpox	0	0	0	0	0	0	0
Smallpox	0	0	0	0	0	0	0
Measles	0	0	0	0	0	0	0
Mumps	0	0	0	0	0	0	0
Infectious mononucleosis	0	0	0	0	0	0	0
Infectious hepatitis	0	0	0	0	0	0	0
Epidemic kerato-conjunctivitis	0	0	0		0	0	0
Poliomyelitis	0	0	0	0	0	0	0
Influenza	0	0	0	0	0	0	0
Common cold	0	0	0	0	0	0	0
Parasite							
E. histolytica	0	0	0	I	U	I	U
Trichomonas vaginalis	0	0	0	I (locally)	U	U	U
Fungus							
Actinomyces bovis	I	I + S.D.	0	II	III	II	U

I–IV = Approximate order of preference. 0 = Ineffective. U = Unknown. S.D. = Sulfadiazine. Strep. = Streptomycin.

aerogenes, *E. coli*, and paracolon bacillus often respond to sulfonamide therapy. For this reason, in vitro sensitivity tests are indicated in such infections. The sulfonamides may be used instead of penicillin in pneumococcal pneumonia, in many beta hemolytic streptococcal infections and in anthrax. They are commonly combined with penicillin in Gram positive coccal infections. They may be used together with chloramphenicol, chlortetracycline or streptomycin in *H. influenzae* meningitis. Brucellosis can be successfully treated with streptomycin plus sulfadiazine. However, recent work indicates that chlortetracycline or oxytetracycline plus streptomycin may be more effective. Gantrisin is now the preferred sulfonamide by many clinicians because of the infrequency of crystalluria and almost complete absence of renal complications (Brickhouse *et al.*, 1949). Furthermore, adjuvant alkali therapy is not needed. Mixtures of 2 or 3 sulfonamides are also effective in reducing intrarenal precipitation of these drugs because of the increased solubility of the mixture. The solubility of the mixture is approximately equal to the sum of the solubilities of components (Lehr, 1945). The insoluble sulfonamides (sulfasuxidine and sulfathalidine) are recommended for treatment of intestinal infections such as ulcerative colitis, regional ileitis and bacillary dysentery. These latter drugs are still considered among the best for the preoperative preparation and postoperative therapy of intestinal surgery patients.

Penicillin is preferred in streptococcal, gonococcal, pneumococcal and spirochaetal infections including syphilis, in rat bite fever and in anthrax. In vitro sensitivity tests should be done for all strains of alpha hemolytic and nonhemolytic streptococci isolated from subacute bacterial endocarditis and in the management of all serious staphylococcal infections. Penicillin should not be used instead of specific antitoxin in diseases in which exotoxins play a major part, i.e., diphtheria, gas gangrene or tetanus. It may be a valuable adjunct in therapy of such cases and is particularly effective in eliminating the carrier state in diphtheria. We have recently shown that oral penicillin prophylaxis is effective in the prevention of rheumatic fever recurrences (Kohn *et al.*, 1953). The use of oral penicillin for treatment of severe systemic infections is not recommended.

Dihydrostreptomycin offers no special advantages over streptomycin since it can cause permanent auditory nerve damage while streptomycin gives rise to vestibular dysfunction. Combined therapy with streptomycin and penicillin is sometimes successful in curing bacterial endocarditis due to *Strep. fecalis*, which may fail to respond to large doses of

either drug alone. I have already mentioned that intestinal infections due to sensitive strains of *Proteus* spp., *Ps. aeruginosa* or paracolon organisms can be successfully treated with oral streptomycin (Milzer *et al.*, 1952). The quantity of streptomycin absorbed through the gastrointestinal tract is negligible.

The tetracyclines have a wide antibacterial spectrum and are clinically effective in a large variety of infections due to both Gram positive and Gram negative bacteria, all of the rickettsiae and a few of the larger viral agents. Tetracycline is preferred for serious staphylococcal infections due to penicillin resistant strains, tularemia, brucellosis, granuloma inguinale, psittacosis and atypical pneumonia. However, only cases of atypical pneumonia associated with a significant rise in cold agglutinin titer apparently respond (see Chapter 10). Chlortetracycline appears to be as effective as penicillin in anthrax and some of the coccal infections, especially *Strep. fecalis*. It is of particular value in urinary tract infections due to sensitive strains of *E. coli*, *A. aerogenes* and other Gram negative bacteria. It is very promising for treatment of intestinal amebiasis and appears effective in pertussis, gonorrhea and syphilis but further work must be reported before a definite recommendation can be made. Finally, chlortetracycline is of great value in the prophylaxis and treatment of peritonitis.

Chloramphenicol is especially effective in the Gram negative bacillary and rickettsial infections. It is not as effective as chlortetracycline in pneumococcal and hemolytic streptococcal infections. Chloramphenicol is the drug of choice for typhoid and is as effective as chlortetracycline or oxytetracyline in brucellosis, *Shigella* infections, tularemia, rickettsial infections, psittacosis, lymphogranuloma venerum and certain cases of atypical pneumonia. Its principal use in in urinary tract infections due to sensitive strains of Gram negative bacilli, particularly members of the colon-aerogenes group. Recent evidence indicates that combined with sulfadiazine it may be the drug of choice for *H. influenzae* meningitis.

Erythromycin is active against Gram positive bacteria in low concentrations and certain Gram negative organisms. It also has been reported to be effective against rickettsia and the large viruses. Erythromycin has a bacterial spectrum very similar to that of penicillin and is more effective against streptococci and pneumococci than the other broad spectrum antibiotics. At present it is the drug of choice for therapy of infections due to penicillin resistant strains of *Staphylococcus aureus*.

Bacitracin, polymyxin, and neomycin were not included in Tables 9.5 and 9.6 because they may produce severe renal injury when administered

systemically. Bacitracin's antibacterial spectrum is very similar to that of penicillin, and it is used principally for treatment of superficial infections such as infected wounds, ulcers, etc. It is recommended for topical application in individuals who have infections due to penicillin-resistant organisms or are allergic to penicillin.

Polymyxin B is the most effective drug at present against serious *Ps. aeruginosa* infections which do not respond to the less toxic antibacterial agents. Most strains of *Ps. aeruginosa* are resistant to the sulfonamides and antibiotics including chlortetracycline, chloromycetin and oxytetracycline but are very sensitive to polymyxin. It is also very effective against the other Gram negative bacilli except *Proteus* spp. which are resistant. Polymyxin B should be considered, therefore, in infections due to other sensitive Gram negative bacilli which are resistant to the less toxic agents. Polymyxin D produces renal damage more readily than polymyxin B and should not be used. On the other hand, the principal evidences of toxicity of Polymyxin B are neurologic and allergic reactions which are usually of a mild degree and transient (Kagan *et al.*, 1951).

The toxic reactions following the administration of penicillin, streptomycin or dihydrostreptomycin are well known and will not be repeated. The important reactions produced by chlortetracycline include nausea, vomiting and loose stools, often amounting to a diarrhea. Milk or the antacid carmethose are effective in overcoming the epigastric distress caused by chlortetracycline without affecting the blood level, but amphojel (aluminum hydroxide gel) should not be used because the gel significantly diminishes the absorption of chlortetracycline from the intestine (Greenspan *et al.*, 1951). Localized dermatitis has been reported following the use of chlortetracycline ointment. Drug fever is rarely seen (Long *et al.*, 1950). Chloramphenicol also produces nausea, vomiting and loose stools, but to a less degree than chlortetracycline. Granulocytopenia, aplastic anemia, a scaly skin eruption and drug fever have been reported following treatment. Oxytetracycline gives rise to nausea, vomiting and diarrhea similar to that produced by chlortetracycline and chloramphenicol. Skin eruptions and drug fevers have also been encountered. Oxytetracycline, chlortetracycline and chloramphenicol can all cause irritation of the mouth and tongue with soreness and ulceration. Monilia infections of the mouth, vagina, anal region, etc. are often observed in patients receiving large doses of these three antibiotics. Complications induced by the antimicrobial agents have recently been reviewed by Finland *et al.* (1953b).

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CHAPTER 10

DIAGNOSIS OF VIRAL AND RICKETTSIAL DISEASES

By Albert Milzer

GENERAL CONSIDERATIONS

Viruses may be defined as ultramicroscopic agents that require living cells for propagation. The viruses usually are filterable through filters whose pores retain bacteria. On the other hand, the rickettsiae are visible with the optical microscope but also require living cells for propagation. The rickettsiae are often regarded as microorganisms intermediate in characteristics between bacteria and viruses. In tissue cultures the viruses multiply at an optimum rate in cells which are actively growing and metabolizing, while the rickettsiae proliferate best in slowly growing cells. Except for Q fever (*Coxiella burnetii*) the rickettsiae are not usually filterable and are transmitted principally by arthropods. A classification of the common human viral and rickettsial diseases is given in Tables 10.1 and 10.2.

In general the basic principles underlying the diagnosis of the bacterial infections also apply to the viral and rickettsial diseases. Three types of laboratory examinations are employed for diagnosis of viral and rickettsial diseases: (1) microscopic examination, (2) various serologic tests to detect the presence of specific antibodies, and (3) attempts to isolate the etiologic agent by chick embryo, animal, and more recently, tissue culture inoculation. Of these three procedures, microscopic examination and the serological tests are the most practical. The usefulness of the microscopic examination is extremely limited as will be discussed while the serologic approach depends on the demonstration of a significant rise (fourfold or greater) in antibody titer in serums obtained during the acute and convalescent phases of illness. Serologic tests include the complement fixation, hemagglutination inhibition and neutralization tests. The disadvantage of the serologic method is that a diagnosis can usually be made only after the disease is well established rather than in the early stages of illness.

Recently serologic tests for many of the viral and rickettsial diseases have been developed to the point that hospital laboratories equipped to carry out complement fixation tests can perform many of these tests routinely and be of great diagnostic aid to the clinician. Also commercial diagnostic antigens and antiserums are now available for many vi-

TABLE 10.1

Classification of Common Human Viral Diseases

I. Dermatotropic (skin lesions)
- Smallpox
- Vaccinia
- Chickenpox
- Herpes simplex
- Herpes zoster
- Molluscum contagiosum
- Verruca
- Inclusion conjunctivitis
- Trachoma
- Epidemic keratoconjunctivitis

II. Neurotropic (CNS lesions)
- Lymphocytic choriomeningitis
- Poliomyelitis
- Rabies
- Equine encephalomyelitis (Eastern & Western type)
- St. Louis encephalitis
- Japanese B encephalitis
- Russian Spring-Summer encephalitis

III. Respiratory
- Influenza A, A-prime, B & C
- Psittacosis & ornithosis
- Common cold (?)
- Atypical pneumonia

IV. Viscerotropic (generalized infections)
- Mumps
- Yellow fever
- Measles
- Lymphogranuloma venereum
- Infectious hepatitis
- Serum jaundice
- Infectious mononucleosis
- Coxsackie virus

ruses and rickettsiae. The serologic procedures which are chiefly applicable to routine work include the complement fixation and hemagglutination inhibition tests which are discussed in detail. The neutralization test is very useful and fairly accurate but is too technical, costly and time-consuming for routine diagnostic use.

Animal or chick embryo inoculation is too expensive and impractical for the average hospital laboratory for several reasons. The number of positive isolations is relatively low and highly trained personnel are needed to identify the isolated agents. There is also considerable risk of laboratory infection in working with many of the viruses and rickettsiae in the active state. Many of the viruses, especially of the neurotropic group, are present in the blood or other blood fluids only during the incubation period or acute phase; by the time the physician sees the patient the virus may no longer be present and the chances of isolation are minimal.

Another disadvantage of animal or chick embryo inoculation for virus isolation is that certain stocks of laboratory animals are known to be spontaneously infected with viruses which might be confused with the strain isolated from the patient. These include lymphocytic chorio-

TABLE 10.2

Classification of Human Rickettsial Diseases

Immunological Group	Etiologic Agent	Principal Arthropod Vector
I. Spotted fever		
Rocky Mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor andersoni* *D. variabilis*
Boutenneuse fever	*R. conori*	*Rhipicephalus sanguineus*
Rickettsialpox	*R. akari*	*Allodermanyssus sanguineus*
II. Typhus		
Epidemic typhus	*R. prowazeki*	*Pediculus humanus*
Murine typhus	*R. mooseri*	*Xenopsylla cheopis* *Polypax spinulosa*
III. Tsutsugamushi disease or scrub typhus	*R. orientalis*	*Thrombicula akamushi* *T. deliensis*
IV. Q fever	*Coxiella burnetii*	*Haemaphysalis humerosa* *D. andersoni* *Amblyomma americanum*

meningitis, Theiler's encephalomyelitis virus, mouse pneumonitis, etc. In fact, the normal chick embryo has been found infected with the virus of Newcastle disease and a pleuropneumonia-like organism. Despite these disadvantages some of the larger laboratories may on occasion receive requests to isolate such viruses as influenza or herpes simplex which can be carried out by chick embryo inoculation or diagnosis of rabies by smears for Negri bodies or mouse inoculation. For this reason virus isolation and identification technics which are applicable to these diseases will be discussed in some detail; the primary purpose of this chapter, however, is to stress those procedures which can be carried out in the hospital laboratory as well as the collection and shipment of specimens to specialized laboratories.

COLLECTION OF SPECIMENS

Serologic Tests. Blood specimens for complement fixation, hemagglutination inhibition or neutralization tests must not be frozen nor

should anticoagulants be added. Draw at least 15 to 20 cc. of blood from the fasting patient with a dry, sterile syringe and allow the blood to clot at room temperature for $\frac{1}{2}$ hour. Centrifuge at 2500 r.p.m. for 20 minutes and pipette the clear serum into pyrex Wassermann tubes with rubber stoppers. Whole blood must not be refrigerated before removing the serum if the cold agglutination test is to be done. Seal the stoppers with adhesive tape as a further precaution against leakage. Carefully label each tube with the *patient's name*, *clinical diagnosis*, *physician's name*, *date of onset* and *date drawn* using adhesive tape or a paper label covered with Scotch tape. Hemolyzed serum is not suitable for the complement fixation test. Serum specimens for the complement fixation test may be stored temporarily in screw-capped tubes in the refrigerator at 4 to 10°C. for 3 weeks (Schmidt *et al.*, 1954). Sera to be stored for as long as 6 weeks should be placed in flame-sealed glass ampules and kept in the refrigerator or dry ice (solid carbon dioxide) freezer.

For the proper interpretation and evaluation of results two or more serial specimens of serum should be tested for the presence of antibodies as follows:

1. Early during the acute phase of illness
2. Two to four weeks after onset (influenza 10 to 14 days)
3. If necessary, later during convalescence (4 to 8 weeks after onset)

In some instances additional specimens must be taken during convalescence because of the relatively slow development of neutralizing antibodies, e.g., lymphocytic choriomeningitis (see Table 10.4).

Virus Isolation. Material for virus or rickettsial isolation must be obtained as soon as possible after onset of illness because these agents become increasingly more difficult to detect later in the course of the disease. Pathologic specimens from which viruses may be isolated include blood, pus, sputum or respiratory secretions, spinal fluid, biopsy tissue and feces depending on the particular disease suspected. Viruses may also be isolated from various tissues obtained at autopsy. A chart summarizing the detection of viruses in various human tissues, secretions or excretions is shown in Fig. 10.1. The various specimens for virus studies are collected as follows:

1. *Nasopharyngeal washings.* Collect nose and throat washings in a sterile container by gargling with 10 to 20 ml. of sterile nutrient broth or isotonic salt solution for several minutes. For young or uncooperative children, introduce a few ml. of solution into the nasal passages with a syringe or use a nose and throat swab.
2. *Blood.* Draw 15 to 20 ml. of whole blood with a sterile syringe

Disease	Brains and Cord	Spinal Fluid	Blood	Respiratory Secretions and Saliva	Conjunctiva	Feces	Skin and Genital Lesions
Lymphocytic choriomeningitis	+	+	+	+	−	+	−
St. Louis encephalitis	+	−	+*	−	−	−	−
Equine encephalomyelitis	+	+*†	+*†	−	−	−	−
Jap. B. encephalitis	+	+	+	−	−	−	−
Poliomyelitis	+	−	+*	+	−	+	−
Rabies	+	−	−	+	−	−	−
Herpes simplex	+	+‡	−	+‡	−	−	+
Lymphogranuloma venereum	−	+	+*	−	+	+	+
Psittacosis	−	−	+	+	−	+	−
Influenza	−	−	−	+	−	−	−
Yellow fever	−	−	+	−	−	−	−
Variola	−	−	−	−	−	−	+
Mumps	−	+	+*	+	−	−	−
Inclusion conjunctivitis	−	−	−	−	+	−	−
Molluscum contagiosum	−	−	−	−	−	−	+
Epidemic keratoconjunctivitis	−	−	−	−	+	−	−
Russian Spring-Summer encephalitis	+	+	+				
Coxsackie virus		+*	+*	+	−	+	−

* Isolated rarely.
† Western type.
‡ Found in normal individuals.

FIG. 10.1. Detection of virus in various human tissues, secretions or excretions

and defibrinate by shaking in a sterile 250-cc. Erlenmeyer flask containing glass beads or by the addition of 1 mg. of heparin to 5 ml. of blood. If this is not possible, whole clotted blood may be submitted. Blood should not be defibrinated with citrate or oxalate because these chemicals may inactivate the virus or are toxic when inoculated intracerebrally into mice. Hemolysis of erythrocytes due to freezing blood does not appear to inactivate viruses or rickettsiae.

3. *Spinal fluid.* Collect at least 5 ml. aseptically.
4. *Autopsy material.* Specimens must be taken as soon as possible after death because of the lability of many of the viral agents as well as the rapid multiplication and spread of bacteria. Obviously, viral studies are useless if the body has been embalmed prior to autopsy. Whenever possible aseptic technic and sterile instruments should be used to remove tissue specimens after first searing the

surface of the organ with a red hot spatula. If an encephalitis virus is suspected, remove the brain with sterile precautions and take small blocks of tissue (about 2 ml.) from the (a) motor cortex (b) hippocampus, (c) midbrain and (d) brain stem. For poliomyelitis take specimens of cervical and lumbar enlargements of spinal cord, medulla, pons and feces from the intestine. For the psittacosis-pneumonitis group, take specimens of the lungs and spleen. Duplicate blocks from each of the above tissues should be fixed in 10 per cent formalin or Zenker's solution or both for pathologic study. Postmortem blood can be taken after searing the surface of the heart with a sterile syringe or pipette.

In the collection of material for virus or rickettsial isolation it must be emphasized that many of these agents are unstable and rapidly become inactivated at room temperature but retain their infectivity if stored at low temperatures. Probably the best technic for preservation until inoculations for isolation can be carried out is storage of infected specimens in dry ice (solid carbon dioxide) at −76°C. Fluid specimens should be placed in sterile pyrex Wassermann tubes or vaccine bottles, while wide-mouth sterile bottles are used for tissues or feces. The containers are sealed with a rubber stopper or screw cap held firmly in place with adhesive tape or melted paraffin, labeled and promptly frozen by immersion in a mixture of alcohol and dry ice. Never fill the glass container more than half full in order to avoid breakage on freezing. Sealed glass ampules are a satisfactory substitute for fluid specimens. The tubes should be rotated during freezing in order to reduce breakage due to fluid expansion and then stored in dry ice pending delivery to the laboratory. It is imperative that the container be tightly sealed against penetration of carbon dioxide gas which may inactivate the virus.

Ordinarily most viruses will not survive more than one or two days at the usual refrigerator temperatures. Those laboratories which wish to preserve viruses and rickettsiae will find a low temperature storage cabinet such as the one described by Horsfall (1940) useful. More recently it has been shown that a number of viruses can be successfully stored in a mechanical refrigerator at −25°C. (Olitsky *et al.* 1949).

If solid carbon dioxide is not available, a less satisfactory procedure applicable to storage of small pieces of infected tissues (about 1 ml.) is by immersion in 50 per cent buffered glycerol (pH 7.4) kept at 3 to 5°C. The advantage of this method is that most pathogenic bacteria which may also be present in the infected tissue do not survive in glycerol longer than five or six days. Many neurotropic viruses will survive for

at least two or more weeks, and poliomyelitis virus infected spinal cords have been preserved for several years by this technic. Psittacosis virus, however, is inactivated by glycerol. The author has found that the addition of one part inactivated normal rabbit serum to nine parts of buffered glycerol significantly increases the survival time of the virus of lymphocytic choriomeningitis.

Drying of viruses and other biologic materials while in the frozen state in a lyophile apparatus is another excellent method for preservation. Such a procedure is rarely practical for the hospital laboratory.

Rickettsia Isolation. Whole blood collected early after onset of febrile illness is the material of choice for isolation. In fatal cases specimens of clotted blood, liver and spleen should be obtained as soon as possible after death.

SHIPMENT OF SPECIMENS

If the hospital laboratory is unable to conduct viral or rickettsial studies, other laboratories set up to carry out such tests which might be contacted include the following:

1. Local City and State Health Departments.
2. University Medical Schools.
3. U. S. Public Health Service, Communicable Disease Center Virus and Rickettsia Laboratory, Montgomery, Ala.
4. U. S. Public Health Service, National Institutes of Health, Microbiological Institute, Division of Infectious Diseases, Bethesda 14, Maryland.
5. Department of Viral and Rickettsial Diseases, Army Medical Department Research and Graduate School, Washington 12, D. C.

Serologic. Serum specimens should be carefully packed in a double mailing container and sent by air mail if arrival can be expected within 36 hours. The package should be labeled **BACTERIOLOGIC SPECIMEN—RUSH** and **FRAGILE.** Serum does not need to be sent in the frozen state. Whole blood should not be shipped because of the likelihood of hemolysis which interferes with the complement fixation test. Be certain to include the *patient's name*, *pertinent clinical* and *laboratory data*, *date of onset of illness* and *date serum drawn* as well as the *physician's name* and *address.* Many laboratories will not accept a single specimen of serum but insist that both acute and convalescent phase serums be submitted simultaneously.

Virus Isolation. It is best to ship specimens frozen in dry ice. Wrap the specimen tubes with cotton or several layers of gauze and pack

carefully in a metal vacuum bottle. Fill the remainder of the vacuum bottle with small pieces of dry ice so that the tubes are in direct contact with dry ice. Cut a small V-shaped slot longitudinally in the vacuum bottle cork and punch a tiny hole in the outer metal cap of the bottle in order to permit the escape of gaseous carbon dioxide. Stopper the bottle and pack carefully in a strong cardboard box. Label the package **BACTERIOLOGIC SPECIMEN—RUSH** and **FRAGILE.** Send by the fastest means available such as air express or special delivery air mail and avoid arrival during the week end. Telegraph the laboratory when the specimen is being sent so that it can be received at the airport if necessary. Specimens prepared and packed as directed above should remain frozen 24 to 36 hours. The following essential information should accompany all specimens: *patient's name, type of specimen, brief clinical history and findings, date of onset, dates of specimens, pertinent laboratory findings, physician's name, etc.*

An alternate method for shipment of specimens in dry ice recommended by the Illinois Department of Public Health consists of the use of insulated bags such as are used for transporting quart packages of ice cream. The specimen bottle is placed in direct contact with dry ice in the insulated bag. The bag containing the ice and specimen jar is in turn packed in a shipping carton containing several inches of insulation (paper, cotton, etc.). Two pounds of dry ice insulated in the above manner should last fifteen tc seventeen hours. In very warm weather or when shipment takes longer, 3 to 5 lbs. of dry ice must be used.

When it is not possible to use dry ice, the second procedure of choice is to ship tissue specimens immersed in at least 10 volumes of 50 per cent buffered glycerol (pH 7.4). This technic is not applicable to psittacosis because, as already mentioned, this virus is inactivated by glycerol.

MICROSCOPIC EXAMINATION

Inclusion Bodies. The application of the optical microscope to the diagnosis of viral diseases is limited because of the similar pathologic lesions that they produce (Fig. 10.2). This is especially true of the neurotropic viruses except rabies in which the Negri body (Plate 10.I) is pathognomonic. A few other viruses also produce characteristic intranuclear or intracytoplasmic structures called "inclusion bodies" within the cells they infect. Viral inclusions may be eosinophilic or basophilic depending on their nature and to some extent the staining procedure

used. Routine staining of tissues fixed in Zenker's fluid with hematoxylin and eosin is usually adequate to demonstrate nuclear inclusions, while special technics are often required to see cytoplasmic inclusions. Usually inclusion bodies approximate the diameter of an erythrocyte, but may range in size from 1 to 20 microns.

Inclusion bodies may be intranuclear, intracytoplasmic or both. The

Disease	Laboratory Animals of Choice	Special Pathology	Serology of Convalescent Serum		
			Neutralization test	Complement fix., test	Other Tests†
Lymphocytic choriomeningitis	Mouse and guinea pig pig	Choriomeningitis	+	+	–
St. Louis encephalitis	Mouse	Encephalitis	+	+	–
Equine encephalomyelitis	Mouse and guinea pig	Encephalomyelitis	+	+	–
Jap. B. encephalitis	Mouse	Encephalitis	+	+	–
Poliomyelitis	Monkey	Degeneration anterior horn cells	+	+ (exp.)	–
Rabies	Mouse	Negri bodies	+	+ (exp)	–
Herpes simplex	Fertile egg, rabbit cornea and mouse	Inclusions, keratitis encephalitis	+	–	–
Lymphogranuloma venereum	Mouse and fertile egg	Encephalitis Miyagawa corpuscles	+	+	–
Psittacosis	Mouse and fertile egg	L.C.L. bodies ‡	+ low titer	+	–
Influenza	Fertile egg, mouse and ferret	Pneumonia	+	+	Red cell aggl.
Yellow fever	Mouse and monkey	Encephalomyelitis liver necrosis—(Councilman inclusions)	+	+*	Ppt. test*
Variola	Fertile egg and rabbit cornea	Guarnieri bodies	+	+	Ppt. Test
Mumps	Fertile egg and monkey	Parotitis	+	+	Red cell aggl.
Inclusion conjunctivitis	—	Smear for inclusions	–	–	–
Molluscum contagiosum	—	Smear for inclusions	–	–	–
Epidemic keratoconjunctivitis	Mouse	Encephalitis	+	–	–
Russian Spring-Summer encephalitis	Mouse	Meningo-encephalomyelitis	+	+	–
Coxsackie virus	Suckling mouse and suckling hamster	Degeneration skeletal muscle	+	+ (exp.)	–

* Significance of test controversial.

† Ppt. = Precipitation test.

‡ LCL (Levinthal, Coles & Lillie) bodies are elementary bodies regarded as the virus.

FIG. 10.2. Identification of etiologic virus

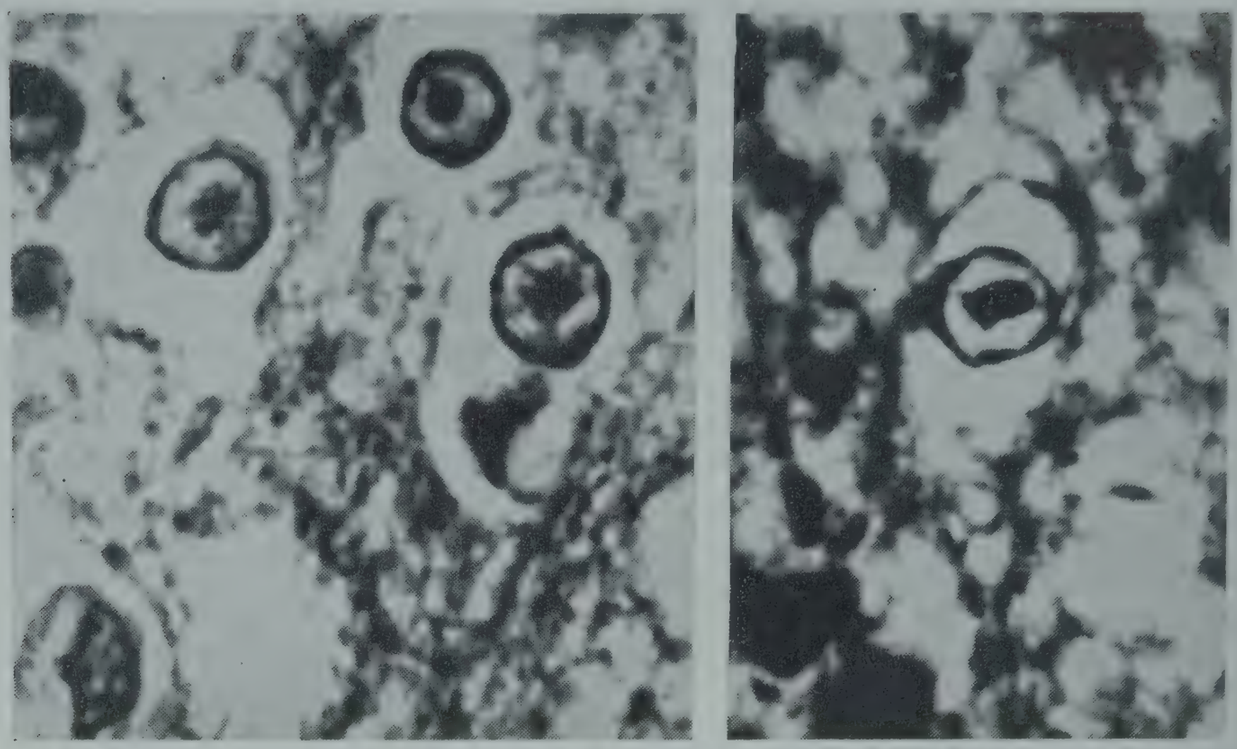


FIG. 10.3. Intranuclear herpetic inclusion bodies in human brain. Phloxine and methylene blue strain; ×1075. From Zarafonetis *et al.*, 1944. Reproduced through the courtesy of the authors.

Guarnieri body is cytoplasmic and nuclear in smallpox and only cytoplasmic in vaccinia. Intranuclear inclusion bodies are found in herpes simplex (Fig. 10.3) and herpes zoster; intracytoplasmic inclusion in rabies (Negri body), trachoma, inclusion conjunctivitis (Fig. 10.4), psittacosis and molluscum contagiosum. There is considerable evidence that a number of the cytoplasmic inclusion bodies such as the Guarnieri body in vaccinia are composed of a mass of smaller particles called elementary bodies which are believed to be the virus. Textbooks of pathology and virology may be consulted for illustrations of the various inclusion bodies and staining technics.

It must be emphasized that the majority of viruses do not produce inclusion bodies that are sufficiently characteristic to be diagnostic. Also it is possible to produce very similar if not identical structures (especially intranuclear) by the inoculation of certain inorganic substances and normal brain tissue. Blank *et al.* (1951) have described characteristic giant epithelial cells in scrapings taken from the peripheral portion of vesicles produced by the viruses of herpes simplex, herpes zoster and varicella. These diagnostic cells are not found in vesicular lesions of the skin due to any other cause, including variola and vaccinia. Addition virus studies are needed, however, to differentiate between herpes simplex and herpes zoster. Variola and vaccinia cannot be differentiated by microscopic examination alone.

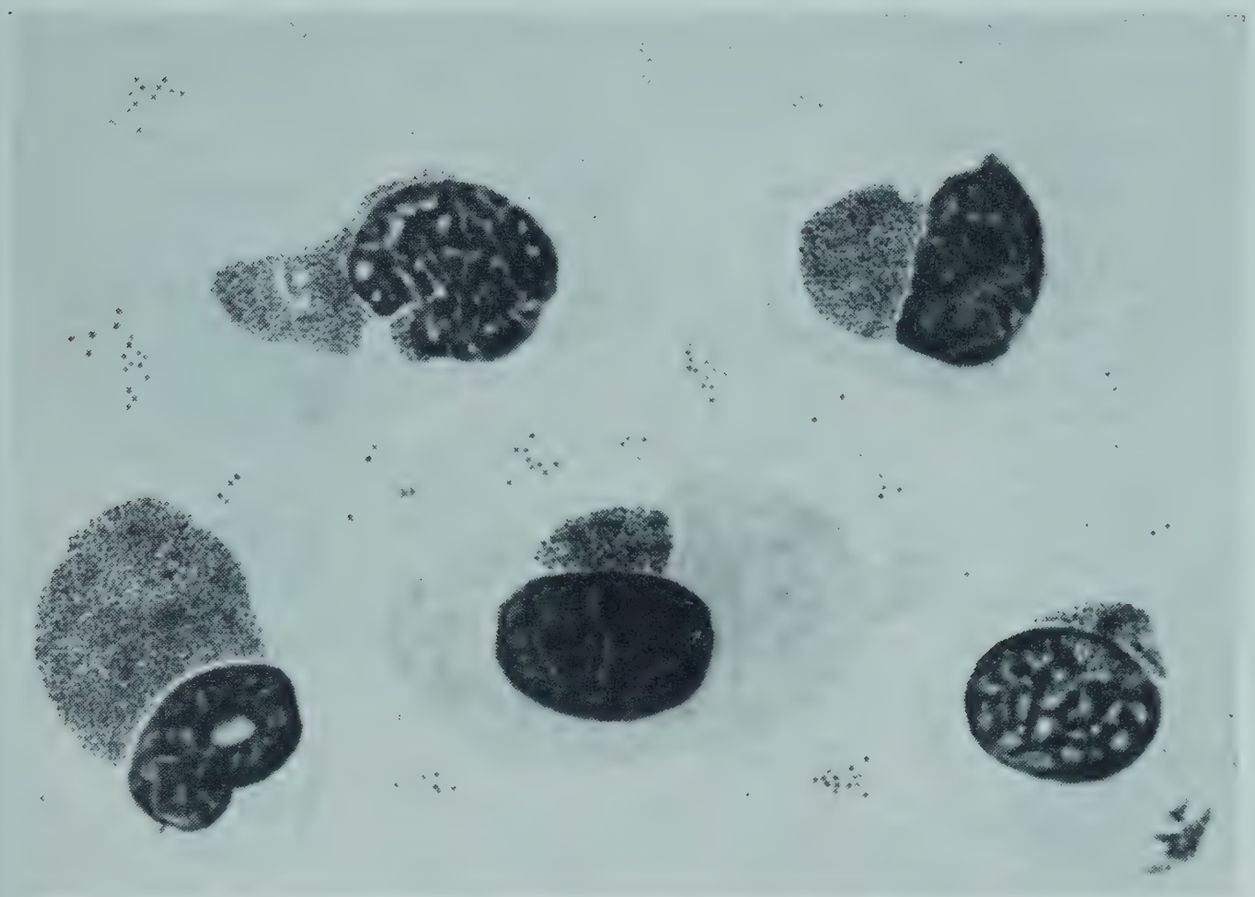


FIG. 10.4. Inclusion bodies of inclusion conjunctivitis. Giemsa strain; approximately ×560. From Thygeson, 1943. Reproduced through the courtesy of the author.

Rickettsiae. The rickettsiae stain poorly with the usual bacteriologic stains but stain red by Macchiavello's method or pinkish purple with Giemsa stain. They are pleomorphic, coccobacillary in form and vary considerably in size from almost invisible particles to 2 microns in length. Rickettsiae develop in the cytoplasm of the cell, except Rocky Mountain spotted fever rickettsiae which are also frequently seen in the nucleus. Large serosal cells with many rickettsiae crowded in the cytoplasm (Neill-Mooser bodies) are seen in the tunica vaginalis of male guinea pigs infected with murine typhus. Specific rickettsial infections, however, cannot be diagnosed with certainty by microscopic examination.

Electron Microscopy. The stained elementary bodies of only a few of the larger viruses such as vaccinia and psittacosis are visible with the optical microscope. Most viruses are too minute for its resolving power. On the other hand, stained rickettsiae are readily visible. A chart depicting the enormous range of size of the viral agents is shown in Figure 10.5.

Although the electron microscope can be used successfully to demonstrate the size and shape of many of the smaller viruses, it is impractical for routine diagnostic work at present. First the cost of this instrument

APPROXIMATE SIZES OF VIRUSES AND *REFERENCE MATERIALS*

	Diameter or width X length in mμ
Red blood cells	7500
B. prodigiosus (*Serratia marcescens*)	750
Rickettsia	475
Psittacosis	450
Canary pox	260 x 310
Myxoma	230 x 290
Vaccinia	210 x 260
Pleuro-pneumonia organism	150
Pseudo rabies	150
Herpes simplex	150
Rabies fixe	125
Influenza	115
Newcastle disease	115
Vesicular stomatitis	100
Staphylococcus bacteriophage	100
Fowl Plague	90
T_2 coli bacteriophage	60 x 80
Chicken tumor I	70
Equine encephalomyelitis	50
T_3 coli bacteriophage	45
Rabbit papilloma (Shope)	44
Pneumonia virus of mice	40
Tobacco mosaic and strains	15 x 280
Latent mosaic of potato	10 x 525
Gene (*Muller's est. of max. size*)	20 x 125
Southern bean mosaic	31
Rift valley fever	30
Tomato bushy stunt	26
Poliomyelitis (Lansing)	25
Hemocyanin molecule (*Busycon*)	22
Yellow fever	22
Hemocyanin molecule (*Octopus*)	20
Louping ill	19
Tobacco ring spot	19
Japanese B encephalitis	18



FIG. 10.5. Approximate sizes of viruses and reference materials. From Stanley, 1947. Reproduced through the courtesy of the author.

is prohibitive for most laboratories, and an ultracentrifuge is needed for the preparation of specimens. Secondly, tissue particles and other artifacts closely resembling published virus electron micrographs may be seen in preparations of normal tissues of the same type used to propagate the virus. Finally, normal body fluids also frequently contain particles and artifacts closely resembling viral agents.

DIAGNOSIS OF RABIES

Human infection is usually acquired from the bite of a rabid dog or cat, rarely from wild animals such as squirrels, foxes, skunks, bats, etc. Diagnosis is based on finding Negri bodies in the brain of suspected animals, by inoculation of mice with brain material, or both. Indications for specific post-exposure rabies treatment suggested by the WHO Expert Committee on Rabies (1954) are reproduced in Table 10.3. Microscopic examination will be considered here; mouse injection is discussed in the section on animal inoculation for virus isolation in this chapter. When a dog is suspected of having rabies it is best not to kill the animal but to keep it under observation until it becomes moribund or dies. Negri bodies are seldom found in animals killed during the early stages of the disease. If the animal is infected, it will generally show signs of rabies and die within three to six days. Rabies may practically be excluded if the animal remains alive for fifteen days. If the dog must be killed, do not injure the brain because it is needed for laboratory examination.

As soon as the animal is dead, remove its entire head at once and deliver it promptly to the laboratory. For shipment the standard procedure is to place the head in a water-tight metal container such as a tin can which is placed in a second water-tight metal container with ordinary ice packed around it. It is much better to ship the head frozen in dry ice or better still to remove the brain as described in the section on collection and shipment of specimens. A **FULL HISTORY** of the case is prepared and sent with the package to the nearest state health department. Shipment can be made only by Railway express because the United States postal regulations prohibit conveyance of such specimens by mail, and the package must be plainly labeled: **CAUTION—RABIES SUSPECT—DOG HEAD, RUSH.**

Negri Bodies. Microscopic examination of impression smears stained with Seller's stain for Negri bodies are made from Ammon's horn (hippocampus major), the cerebral cortex and the cerebellum. Negri bodies, as a rule, are most abundant and typical in Ammon's horn (Fig. 10.6),

Table 10.3

*Indications For Specific Post-Exposure Rabies Treatment**

Nature of Exposure	Conditions of Biting Animal		Recommended Treatment
	At time of exposure	During observation period of 10 days	
I. No lesions; indirect contact only	Rabid	—	None†
II. Licks:			
(1) Unabraded skin	Rabid	—	None†
(2) Abraded skin and abraded or unabraded mucosa	(a) Healthy	Healthy	None
	(b) Healthy	Clinical signs or rabies or proven rabid	Start vaccine at first signs of rabies in animal
	(c) Signs suggestive of rabies	Healthy	Start vaccine immediately; stop treatment if animal is normal on 5th day after exposure‡
	(d) Rabid, escaped, killed or unknown	—	Start vaccine immediately
III. Bites:			
(1) Simple exposure	(a) Healthy	Healthy	None
	(b) Healthy	Clinical signs of rabies or proven rabid	Start vaccine at first signs of rabies in animal
	(c) Signs suggestive of rabies	Healthy	Start vaccine immediately; stop treatment if animal is normal on 5th day after exposure‡
	(d) Rabid, escaped, killed, or unknown; or any bite by wolf, jackal, fox, or other wild animal	—	Start vaccine immediately
(2) Severe exposure (multiple; or face, head or neck bites)	(a) Healthy	Healthy	Hyperimmune serum immediately; no vaccine as long as animal remains normal
	(b) Healthy	Clinical signs of rabies or proven rabid	Hyperimmune serum immediately; start vaccine at first sign or rabies
	(c) Signs suggestive of rabies	Healthy	Hyperimmune serum immediately, followed by vaccine; vaccine may be stopped if animal is normal on 5th day after exposure
	(d) Rabid, escaped killed, or unknown. Any bite by wild animal	—	Hyperimmune serum immediately, followed by vaccine

* Reproduced from the WHO Tech. Report Series No. 82. Expert Committee on Rabies. Second Report, 1954.

† Start vaccine immediately in young children and in patients where a reliable history cannot be obtained.

‡ An alternative treatment would be to give hyperimmune serum and not start vaccine as long as the animal remained normal.

Note: To be effective hyperimmune serum must be given wtihin 72 hours of exposure. Dose: 0.5 ml. per kg. of body-weight.

These indications apply equally well whether or not the biting animal has been previously vaccinated.

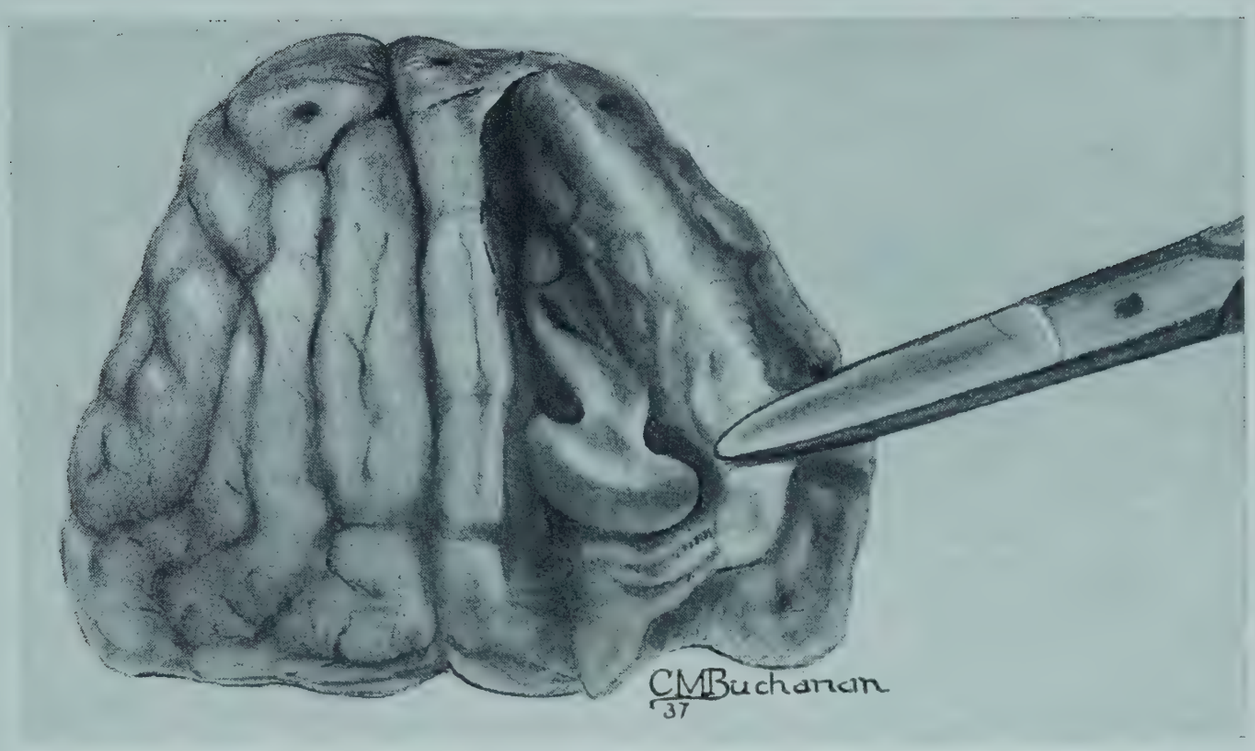


FIG. 10.6. Showing method of incision of dog's brain and exposure of hippocampus major (Ammon's horn) in examination for rabies. Brain is actual size, weighs about 60 grams, and is taken from a large dog.

although they may be present in other portions of the brain. The impression method is recommended because there is relatively little distortion and rupture of nerve cells. Histological sections are rarely necessary except for confirmation in doubtful cases.

Stained preparations are examined first under low power in order to find thin areas with numerous nerve cells, and the detailed structures are sought with the oil immersion lens. Negri bodies are refractive, round or oval intracytoplasmic structures about 3 to 10 micra in diameter which stain red with blue granules (Plate 10.I). The cytoplasm of nerve cells stains blue to purplish-blue, the nuclei and nucleoli deep blue, and the erythrocytes yellow. Stroma stains rose pink and nerve fibers deep pink. Neural sheaths do not stain. At least 50 nerve cells in each preparation should be examined.

A possible source of error in the examination of dog brains is the presence of distemper virus inclusion bodies that are similar to Negri bodies. The distemper inclusion bodies differ in that they are indefinite, pale red and have no inner structure. Normal mice sometimes also have intracytoplasmic inclusion bodies in the brain which are small, pink to bright red, uniformly round and have no inner structure. Since Negri bodies are not demonstrable in about 10 per cent of dogs with rabies, mouse inoculation for virus isolation should be done in all doubtful cases.



PLATE 10.I. Nerve cells in spreads from Ammon's horn. Magnification 1200 diameters.

Figs. 1 and 2 from dog "street rabies" show "Negri bodies" (*NB*); Fig. 3 from non-rabid cat, and Fig. 4 from dog distemper show indefinite inclusion (1) that might be mistaken for Negri bodies by the inexperienced. Negri bodies are structured, more intensely staining, and more refractive. Figs. 1, 3, and 4 are stained by fuchsin-methylene blue mixture, which stains Negri bodies (*NB*) red with blue granules, nucleus (*NUC*) of nerve cells blue, and red-blood cells yellow (*RB*). Fig. 2 is stained by Giemsa's mixture, which stains Negri bodies a robin's egg blue with red granules, nucleus of nerve cells red, and red-blood cells salmon pink. (From Parke and Williams, *Bacteriology*, Lea and Febiger.)

TABLE 10.4

*Average Human Immunologic Pattern in Certain Virus Infections**

Virus	Type Test	Weeks after Onset						1 yr.	2 yrs.
		0–1	1–2	2–3	3–4	7–8	15–16		
Western equine	Neutralization	++	++++	+++++	+++++	+++++	+++++	++	+
	Complement fixation	0	±	+	++	+++	++	±	0
St. Louis	Neutralization	0	±	+	++	+++	++++	++	+
	Complement fixation	±	+	+++	++++	++++	++++	+	±
Japanese B†	Neutralization	±	++	+++	+++	++++	++++	++	+
	Complement fixation	±	++	+++	++++	+++	+++	+	±
Lymphocytic choriomeningitis	Neutralization	0	0	±	±	+	++	+	+
	Complement fixation	0	+	++	++	++	++	±	0

* Data from Hammon, 1948.

† Corrected through the courtesy of Hammon on the basis of more recent data

COMPLEMENT FIXATION TEST

Until recently the value of the complement fixation test for the routine diagnosis of viral and rickettsial diseases has been discredited. With the recent development of purified, specific, non-infectious and commercially available diagnostic antigens the complement fixation test has been used successfully for routine diagnosis of viral and rickettsial diseases in my laboratory for the past few years. Satisfactory complement fixing antigens may be obtained from the Markham Laboratories of Chicago or the Lederle Laboratories. This test has several obvious advantages over virus isolation and the neutralization test. Animals are not required, and in many instances specific complement fixing antibodies can be detected in the patient's serum relatively early in the course of the disease (2–3 weeks after the onset, Table 10.4). For example, complement fixing antibodies for St. Louis encephalitis and lymphocytic choriomeningitis appear much earlier than the neutralizing antibodies. On the other hand, the neutralizing test for equine encephalomyelitis is positive before the complement fixation test. At present the complement fixation test is being successfully applied to the laboratory diagnosis of poliomyelitis by the research laboratory using antigens prepared from virus grown in tissue culture (Svedmyr *et al.*, 1953). The technic has not yet been developed to the point where it can be used routinely by the hospital laboratory.

The complement fixation test is relatively inexpensive, and a technician familiar with the basic principles of this test as practiced in bac-

teriology with proper additional training should be able to use it successfully for routine viral and rickettsial diagnosis. The complement fixation test as applied to viral and rickettsial antigens must be very carefully controlled. Normal tissues of the same source used to grow the virus or rickettsia must be used as antigen controls in each test in order to rule out nonspecific reactions. Also, known positive and negative control serums should be included in each test. The patient's serum must be inactivated at 60°C. for 20 minutes to reduce nonspecific reaction (slightly anticomplementary or Wassermann positive). Kempf *et al.* (1954) have shown that comparable titers are obtained in mumps complement fixation tests regardless of whether the sera are heated at 56 or 60°C. for 30 minutes. Often the serums may still be unsatisfactory unless heated twice at 60°C. or once at 65°C. for 20 minutes. It is advisable to test all positive serums at least twice until considerable experience has been gained in performing the complement fixation test and, if possible, to check positive results by another test such as the neutralization test.

The technic of the complement fixation test used is essentially a modified Kolmer test (Schubert *et al.*, 1951) as recommended by the respective manufacturer of the antigen. First the serum sample of the patient is inactivated by heating at 60°C. for 20 minutes. The test consists of mixing one unit of antigen (0.1 ml.) with two full units of complement (0.2 ml.) and 1:5 and 1:20 dilutions of the serum (0.2 ml.) to be tested. Lower dilutions of the test serum are not used in the screen test in order to avoid cross-fixations. The mixture is incubated in a waterbath at 37°C. for one hour and twenty minutes or overnight at ice box temperature. Next the hemolytic system consisting of 0.4 ml. of an equal volume of 2 per cent suspension of washed sheep cells and amboceptor (hemolysin) solution containing two hemolytic units is added. The tests are read after further incubation at 37°C. for a half hour. The test is repeated with higher dilutions if the 1:5 and 1:20 serum dilutions show 4 plus reaction. An antigen control prepared from normal tissue of the same source used to prepare the antigen, and both positive and negative control serums are included in each test. Anticomplementary reactions of the patient's serum are occasionally encountered. These effects are usually due to bacterial contamination, syphilis or very lipid sera (Lennette, 1954). The presence of various chemical contaminants in the serum such as alcohol, anticoagulants, preservatives, etc. may also give rise to anticomplementary effects. If serums cannot be tested shortly after collection, they may be kept in the refrigerator at 4°C. for 3 to 6 weeks (Schmidt *et al.*, 1954).

HEMAGGLUTINATION INHIBITION TEST

Hemagglutination inhibition is a useful test that can be readily employed in the routine diagnostic laboratory for diagnosis of influenza A and B and mumps virus infections. This test depends upon the fact that allantoic fluid of chick embryos infected with these viruses causes agglutination of chick erythrocytes and those of certain other species when mixed in a test tube and incubated at room or refrigerator temperature. Serums containing antibodies for these viruses will specifically inhibit the hemagglutination in a quantitative manner. Diagnosis depends upon the demonstration of a significant rise in antibody titer between the acute and convalescent phase serums.

Several satisfactory technics for performing the hemagglutinin inhibition test are described in the literature, but results obtained in different laboratories are not comparable because there is no standard technic. Salk (1944) has shown that such factors as concentration of erythrocyte suspension, incubation temperature, etc. markedly influence influenza virus hemagglutinin titers. There are also no standard antigens or antiserums available for reference. The Committee on Standard Serological Procedures in Influenza Studies (1950) has recommended a standard test for influenza diagnosis.

In conducting the hemagglutination inhibition test it is important to determine the virus titer in the infected allantoic fluid removed from chick embryos previously inoculated with influenza or mumps virus. The PR8 strain is used for influenza A; FM-1 and Cuppett strains for influenza A-prime; Lee strain for influenza B, and JJ or 1233 strain for influenza C. The virus titer is read as the highest dilution of the infected allantoic fluid showing complete agglutination and is designated one hemagglutinin unit. In the inhibition test serial two fold dilutions of the patient's serum are made and 4 units of virus hemagglutinin are added to each tube. After mixing the serum and virus a constant amount of chick erythrocyte suspension (0.25 per cent) is added to each tube and the tubes are allowed to stand at room temperature for $1\frac{1}{2}$ hours. In tubes without agglutination the cells settle in the bottom of the tube as a small sharply outlined button, while with positive agglutination the cells settle in a diffuse disc with serrated edges (Fig. 10.7). The titer is considered the highest dilution of serum (final concentration) that completely inhibits hemagglutination.

AGGLUTINATION AND PRECIPITATION TESTS

The agglutination test is limited in its application to a few of the larger viruses such as vaccinia and psittacosis and the rickettsiae. Ag-

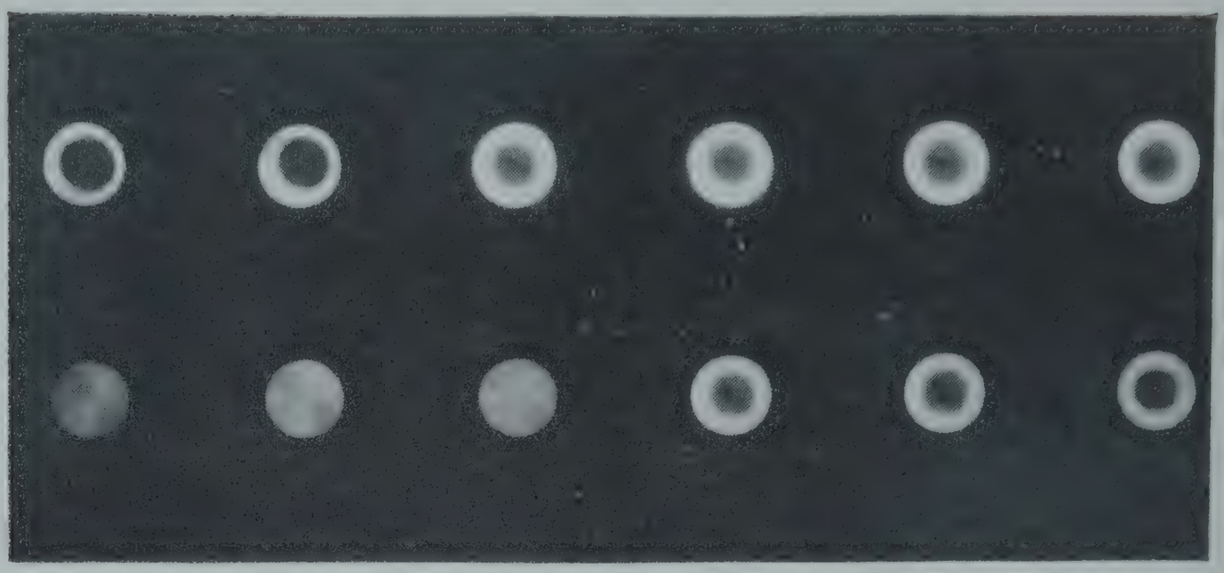


FIG. 10.7. Photograph of bottom view of tubes in a hemagglutination titration test showing patterns formed by sedimented chick erythrocytes in different 2-fold serial dilutions of allantoic fluid infected with mumps virus. The characteristic pattern of complete agglutination of erythrocytes by the mumps virus is seen in the first 3 tubes of the lower row. The remaining tubes are increasing dilutions of the virus and show no hemagglutination. The upper row represents allantoic fluid in which the virus failed to grow and shows no hemagglutination. The last 2 tubes in the upper row are the saline and normal allantoic fluid control tubes.

glutination tests for viruses are similar to those for bacteria, but at present they are not practical for routine work. Rickettsial agglutination tests are satisfactory for diagnosis but are not widely used because of technical difficulties in preparing suitable antigens. The precipitation test is rarely used for the same reason except in smallpox. A ground-up suspension of variolous crusts is specifically flocculated by antivaccinal rabbit serum, and may be used as a practical test for the diagnosis of smallpox (Parker, 1948).

Certain nonspecific tests are available which are of considerable value in diagnosis. These include the heterophile agglutination test for infectious mononucleosis (see Chapter 5), cold hemagglutinins and streptococcus MG agglutinins for atypical pneumonia. The Weil Felix test is of value in the diagnosis of rickettsial diseases (see Chapter 11).

Cold Hemagglutination. Cold agglutinins are antibodies in the serum of certain patients with atypical pneumonia and other diseases which aggregate the patient's erythrocytes or group O erythrocytes at low temperatures (0 to 10°C.) but not at 37°C. Agglutination may also occur at 20° to 25°C. with serums of high titer. These antibodies have no relation to the viral hemagglutinins discussed in the preceding section. Blood drawn for cold agglutinins should not be stored in the refrigerator because the clot will remove these antibodies from the serum. Therefore, the blood is allowed to clot at room temperature, and the

serum is removed before it is stored in the refrigerator until tested. Incubating whole blood at 37°C. will elute the cold agglutinins from the erythrocytes.

The recommended technic for detection of cold hemagglutinins is described by the Commission on Acute Respiratory Diseases (1944):

Serial twofold dilutions of the patient's serum are made in physiological saline solution. To each tube a standard amount of a 0.2% suspension of thoroughly washed Group O erythrocytes is added. After thorough shaking, the tubes are kept overnight at approximately 4°C. After removal from the refrigerator on the following morning, the tubes should be read promptly before warming occurs. Readings are graded from 4+ to 1+. A 4+ reaction consists of a tight disk of cells which does not disperse readily on gentle tapping of the tube; a 1+ is the least amount of definite clumping visible to the unaided eye. The titer is considered highest dilution showing 1+ agglutination. Positive tests must be read for the disappearance of agglutination after 30 minutes incubation at 37°C. in order to rule out heterophile agglutinins.

Streptococcus MG. This is a species of nonhemolytic streptococcus that can be isolated from the respiratory tract or lungs of some cases of primary atypical pneumonia. It has also been isolated from the respiratory tract of normal persons and others with non-pneumonic acute respiratory infections. The role which streptococcus MG plays in the pathogenesis of atypical pneumonia is unknown, but is has been observed that many patients develop agglutinins for this strain during convalescence. The detection of streptococcus MG agglutinins is, therefore, of value in differential diagnosis.

Details of the technic for the streptococcus MG agglutination test are described by Thomas *et al.* (1945):

Several specimens of serum collected in the early acute phase and on a weekly basis should be tested until the diagnosis is made. The antigen consists of a heat-killed washed suspension of an 18-hour broth culture of streptococcus MG adjusted to turbidity No. 5 on the McFarland scale. Merthiolate (1:10,000 final concentration) is added as a preservative. Serial twofold dilutions of *unheated* serum are made in saline solution. Serums are not heated at 56°C. because the agglutination titer is reduced. Each serum dilution is then mixed with an equal volume of streptococcal suspension. The final dilutions of serum should range from 1:10 to 1:320. Dilutions below 1:10 are not tested on account of possible nonspecific agglutination.

The test is incubated in a waterbath at 37°C. for 2 hours, followed by

18 hours in the refrigerator at 4°C. Next the rack is reincubated at 37°C. for 2 hours after which the titer is read as the highest dilution of serum showing visible particles. The incubation period for 2 hours at 37°C. is of importance in ruling out nonspecific agglutination of streptococcus MG which may occur at 4°C. Such agglutination disappears when the tubes are reheated at 37°C.

NEUTRALIZATION TEST

The neutralization test is used to detect the presence of virus-neutralizing antibodies in the patient's serum or may be used to identify an isolated virus provided serum with known specific antibodies is employed. The serum neutralizing antibody is believed to be different from the antibody involved in the complement fixation, agglutination or precipitation reactions. The test serum is mixed with the virus in suitable proportions, incubated and inoculated into susceptible animals or the chick embryo, depending on the virus. Specific neutralizing antibodies render the virus incapable of infecting susceptible cells. No single technic is applicable to all viral agents. In some of these tests the amount of virus used is kept constant and serial dilutions of serum added; in others the amount of serum is kept constant and the virus is diluted. Other variables in the test include the time and temperature of virus-antiserum mixture, age and route of inoculation of the test animal or chick embryo, etc. Enough animals or chick embryos must be used in the test to make the results statistically significant (5 to 10 for each dilution).

Serum should be collected as early as possible during the acute stage and later during convalescence, and a diagnosis is made by the demonstration of a significant rise in specific neutralizing antibody titer. The neutralization test is particularly useful in the diagnosis of the neurotropic virus infections. In performing the test, serial dilutions of acute and convalescent phase serums are set up in parallel and a constant amount of virus is added. Following suitable incubation, the serum-virus mixtures are next injected into susceptible animals or the chick embryo. The 50 per cent end point or LD_{50} (death in half the animals or pathologic lesions) is determined for each. The significance of the difference between the LD_{50} of the acute and that of the convalescent phase serums is measured by the neutralization index, which is the antilogarithm of the difference between the two LD_{50}. This test has not proved to be of equal value for diagnosis of rickettsial infections.

Mice inoculated intracerebrally or occasionally intraperitoneally are

generally used in neutralization tests for the neurotropic viruses except poliomyelitis. The monkey has been used as the test animal in poliomyelitis but the high cost and multiplicity of virus strains make this test impractical for routine diagnostic work. Tissue culture technics using monkey kidney (Enders *et al.*, 1949; Robbins *et al.*, 1951) and the HeLa strain of human malignant cell (Syverton *et al.*, 1954) are now preferred for the primary isolation and the immunological typing of the 3 types of poliomyelitis virus. While these procedures are far superior to the use of the monkey, they have not been developed to the point where they are suitable for routine laboratory diagnosis. The neutralizing antibody for vaccinia and herpes simplex can be titrated by inoculation of suitable virus-immune serum mixtures on the chorioallantoic membrane of the developing chick embryo and counting the number of gross lesions or "pocks" compared to controls inoculated with virus plus normal serum.

The time of appearance of neutralizing antibodies for the various virus infections after onset of disease varies considerably (Table 10.4). Antibodies may be present in significant titer within one week in Western equine encephalomyelitis, but as long as eight to ten weeks may elapse in lymphocytic choriomeningitis before the appearance of antibodies. However, as a rule most virus-neutralizing antibodies are detectable in serum obtained six weeks after onset. Serums containing neutralizing antibodies are best preserved by storage in flame-sealed glass ampules in solid carbon dioxide.

The neutralization test is too technical, costly and time-consuming for routine diagnostic use. It has proved exceedingly useful to confirm positive complement fixation tests for the neurotropic group of viruses. Further information concerning the technic and interpretation of the neutralization test may be found in the excellent articles by Hammon (1948) and Smadel (1952).

ANIMAL INOCULATION

Viral Isolation. It has already been emphasized that isolation and identification of the etiologic viral or rickettsial agent from a patient by animal or chick embryo inoculation is a costly, laborious and time-consuming procedure. Furthermore, one must prove that the virus recovered was isolated from the patient and does not represent a natural or accidental virus infection of the stock animals or a laboratory contamination. The chart in Figure 10.2 summarizes the laboratory animals of choice for isolation and identification of the etiologic virus. In

addition to special histopathologic findings, the serologic tests useful for identification of each virus are indicated in the chart. The following characteristics aid in the identification of the neurotropic viruses by animal inoculation:

1. *Animal host range*—for example, rabies virus is pathogenic for nearly all mammals but poliomyelitis virus only for certain Old World monkeys and the chimpanzee. The Coxsackie virus is pathogenic for only suckling mice and suckling hamsters.

2. *Routes of inoculation that produce disease*—for example, the virus of lymphocytic choriomeningitis can produce infection by almost any route of inoculation, but most strains of poliomyelitis virus must be inoculated intracerebrally or intranasally.

3. *Tissue infected*—for example, the virus of lymphocytic choriomeningitis is found in almost all tissues of the body, but that of St. Louis encephalitis is largely confined to the nervous system.

Specimens uncontaminated by bacteria such as defibrinated blood or spinal fluid may be injected directly by a route likely to produce infection. Tissue specimens, especially if preserved in glycerol, are first washed with sterile distilled water, weighed and cut into small pieces with scissors in a Petri dish. The tissue fragments are then ground in a sterile mortar with enough sterile sand or alundum added to make a paste. Nutrient broth (pH 7.4) or distilled water is added in small amounts (2 to 3 ml.) while grinding, and subsequently the suspension is diluted to a 10 or 20 per cent concentration. Some workers prefer to grind larger pieces of tissues in a special metal container which can be hermetically closed with a screw top and operated on a Waring blendor. Physiologic saline should not be used as the diluent because sodium chloride appears to have a deleterious effect on some viruses. The suspension is centrifuged at low speed (2500 r.p.m. for 10 minutes) to sediment the coarser particles, and the supernatant fluid is removed aseptically for injection.

If the specimen is contaminated (oral washings, stool suspensions, etc.) the procedure of choice for decontamination is the addition of antibiotic mixtures such as penicillin and streptomycin. For sputum, stool suspensions and nasopharyngeal washings, use a final concentration of 500 units per ml. of penicillin and 250 micrograms per ml. of streptomycin. The suspensions are allowed to stand for 30 minutes at room temperature before inoculation. These or higher concentrations may inactivate certain viruses, especially those of the psittacosis-lymphogranuloma group. For this reason, Meyer (1948) recommends

the use of a mixture of tyrothricin, sulfadiazine and streptomycin for isolation of psittacosis virus from contaminated material.

Chemicals have been used to some extent for decontamination but generally are less satisfactory than antibiotics. Ether in 15 per cent concentrations is commonly used to destroy bacteria in stool suspensions for isolation of the poliomyelitis virus (Paul, 1948). Ether, however, inactivates some of the viral agents (Andrewes *et al.*, 1949). Zephiran (1:1000) has been used for the isolation of the influenza virus (Personnel of U. S. Naval Laboratory Research Unit No. 1, 1942).

Filtration through bacteriological filters with a pore size sufficiently fine to retain bacteria is effective for removing these organisms from virus suspensions. This method cannot be recommended for primary isolation because there is always considerable and sometimes a complete loss of virus particles by absorption in the filter. Moreover some of the larger virus particles are not readily filterable even when present in relatively high concentration. The filters commonly used include the Berkefeld, Mandler and Seitz types. More recently the Elford type of graded collodion membrane filter (gradacol membrane) with uniform porosity has superseded the older types of filters in virology laboratories. These membranes have the advantage of being prepared under controlled conditions with uniform pores of known size and are used chiefly in research work.

Diagnosis of Rabies by Mouse Inoculation

The diagnosis of rabies by mouse inoculation should always be carried out in doubtful cases because the use of the Pasteur treatment by the physician often depends on laboratory findings. The technic outlined for isolating rabies virus by intracerebral inoculation of the mouse may also serve as a guide for the procedure one follows in isolating a neurotropic virus.

1. Grind a small portion of Ammon's horn in a sterile mortar and prepare a 10 per cent suspension in broth as described above. Check for bacterial contamination by seeding several tubes of thioglycollate media. In order to save time if contamination is suspected, add penicillin and streptomycin mixture (250 units or micrograms of each per ml.) to the suspension and keep in the refrigerator for 2 hours prior to inoculation. A portion of the suspension should be kept frozen in event the mice are ill on the following day and die of bacterial meningitis and the sterility test shows growth.

2. With a 0.25-ml. tuberculin syringe inoculate 0.03 ml. intracerebrally



FIG. 10.8. Animal inoculation for the diagnosis of rabies. A small amount of emulsified material from Ammon's horn of the suspected rabid animal is injected intracerebrally into a young mouse.

into 4 to 6 week old slightly etherized mice weighing 12 to 14 grams. A strain of albino Swiss mice known to be susceptible to neurotropic viruses as the Webster strain is preferred. A 26- or 27-gauge needle, $\frac{1}{4}$ or $\frac{3}{8}$ inch in length, is employed and the injection is made slightly to one side of the midline and half way between the ear and eye on the same side of the head (Fig. 10.8). No antiseptic is necessary over the site of inoculation, as bacterial infection rarely develops unless the inoculum is contaminated. Secondary infections are infrequent when the brain material has been preserved in 50 per cent buffered glycerol. At least 4 mice should be inoculated with each brain specimen.

3. The earliest signs noticed are sluggishness and ruffling of the fur. This is followed by tremors, convulsions, flaccid paralysis of the legs, prostration and finally death. The onset of central nervous system manifestations usually occurs between the 6th and 8th day after inoculation.

4. Negri bodies can often be demonstrated in the brains of mice on the 5th day after inoculation with *street* rabies virus. After the development of central nervous manifestations, Negri bodies are usually numerous and can be found throughout the brain. The Negri bodies can be demonstrated by etherizing the mouse, removing and making stained preparations of material from the brain. If the microscopic examination is in doubt, the identity of the virus isolated can be checked by cross complement fixation, neutralization or active immunity tests using known rabies immune serum and a standard strain of *fixed* rabies virus.

5. It is advisable to keep the inoculated mice under observation for at least 30 days before discarding. Rarely the incubation period in mice inoculated intracerebrally with positive brain material is prolonged to 2 or 3 weeks.

The isolation of the virus of lymphogranuloma venerum by mouse inoculation is discussed in Chapter 18. Lack of space does not permit further details of animal inoculation for virus isolation, but those interested in this subject may consult publications by Van Rooyen and Rhodes (1948), Rivers (1952) and Francis, Jr. (1948).

Rickettsia Isolation. The male guinea pig weighing 400 to 500 grams is the animal of choice for rickettsia isolation. The same technic is used for isolation of the etiologic agent from the patient in all of the rickettsial diseases. Draw 10 ml. of venous blood as early as possible after onset of the infection and inoculate 5 ml. intraperitoneally into each of two guinea pigs. The blood is best inoculated immediately upon removal from the patient. The guinea pigs should be observed and have their temperature recorded for about one week prior to use in order to be certain that they are healthy. When blood is taken later in the course of the disease (middle or end of the second week of febrile illness), it is advisable to let the blood clot and remove the serum. At that time the serum is likely to contain antibodies that will interfere with the multiplication of the rickettsial agent. The clot is ground in a mortar with sterile alundum or sand, suspended in sterile saline or broth and injected intraperitoneally in 3 to 5 ml. amounts.

The guinea pigs are observed daily and temperatures are recorded. The important findings to be observed in the guinea pig are: (1) the incubation period, (2) the degree and duration of fever, (3) the presence of scrotal swelling, erythema and necrosis, and (4) the severity of illness. Table 10.5 outlines the characteristic findings in this animal. According to Topping (1948) white mice injected intraperitoneally are a satisfactory substitute for the isolation of *Rickettsia orientalis* and *Coxiella burnetii*.

TABLE 10.5

Summary of Rickettsial Infections in Guinea Pigs

Disease	Incubation	Fever	Scrotal Reaction	Other Findings
	days	*days*		
Rocky Mountain spotted fever	3–10	7–12	Swelling and necrosis	Rickettsia in arteriole smooth muscle cells and occasionally intranuclear; usually fatal
Rickettsialpox	6–9	3-5	Swelling and erythema	Ruffled fur and inactivity; moderately enlarged spleen and lymph nodes
Epidemic typhus	5–12	5–6	Absent usually	Low virulence
Murine typhus	4–6	4–6	Swelling and erythema	Neill-Mooser bodies in serosal cells of tunica vaginalis
Tsutsugamushi disease			Absent	Low virulence; some strains very pathogenic
Q fever	3–10	2–8	Absent	Enlarged spleen and lymph nodes; often fatal peritonitis

Once isolated the rickettsiae are maintained by passage in other guinea pigs or in the chick embryo. The rickettsial agent isolated is identified by serologic studies of the serum of convalescent animals or cross-immunity tests. The serologic tests employed include the complement fixation, neutralization and agglutination tests. The reader is referred to Topping (1948) and Pinkerton (1948) for details.

CHICK EMBRYO INOCULATION

Virus Isolation. Considerable progress has been made in recent years in the use of the developing chick embryo for the primary isolation of the viral agents. The chick embryo is also widely used for the manufacture of several viral and rickettsial vaccines and diagnostic antigens for serologic and skin tests. At present chick embryo inoculation is the method of choice for isolation of the following viruses: influenza, mumps, variola, herpes simplex and Newcastle disease. Although many of the neurotropic viruses may be propagated in the chick embryo, animal inoculation is still the procedure of choice for isolation of this group. As already mentioned, although the tissue culture technic appears promising for the primary isolation and immunological typing

of the poliomyelitis virus, this procedure is not suitable for routine use at present. Perhaps the stimulus of these studies will result in further simplification so that the tissue culture method will become more practical for use by the average laboratory for the diagnosis of poliomyelitis as well as other viral diseases in the near future.

Fertile eggs are conveniently obtained weekly from a reliable source with a disease-free flock and incubated at 38 to 39°C. in a humid atmosphere (relative humidity 45 to 60 per cent). The ordinary commercial egg incubator with forced air circulation is satisfactory and inexpensive. The eggs should be turned at least twice daily. Commercial incubators are available which turn the eggs automatically (Humidaire, Model No. 50; New Madison, Ohio). After incubation for about five days the eggs are examined by candling or transillumination for viability. A convenient candler consists of a 100-Watt electric light bulb in a small tin box with an oval opening on one side. The opening is lined with sponge rubber to block all direct light when candling an egg. The candling is done in a dark room. Infertile eggs and dead embryos are discarded. The following criteria aid in determining the viability of the embryo: (1) presence of visible blood vessels, (2) spontaneous movements of the embryo, and (3) absence of undue mobility of the embryo on turning the egg.

Eggs are incubated for five to thirteen days depending on the method of inoculation and the virus to be isolated. Certain viruses such as equine encephalomyelitis and Newcastle disease multiply readily by any route of inoculation and kill the embryo. Other viruses, on the other hand, are best isolated by different routes, i.e., chorioallantoic, allantoic, amniotic or yolk sac inoculation. A tabulation of the preferred routes follows:

1. *Chorioallantoic membrane inoculation* (see Fig. 10.9).
   (a) *Viruses producing readily visible lesions* (plaques)—variola, vaccinia, herpes simplex and Newcastle disease. Chorioallantoic inoculation is recommended for primary isolation of the variola and herpes simplex viruses (Coriell *et al.*, 1949). In my experience two or three egg passages are sometimes required for plaque formation on the chorioallantoic membrane by a recently isolated strain of herpes simplex virus. Other applications include titration of virus suspensions which produce visible lesions, neutralizing antibodies and chemotherapy tests.
   (b) *Viruses producing insignificant or no lesions*—influenza, psit-

tacosis-lymphogranuloma group, yellow fever, St. Louis encephalitis, Japanese B encephalitis and lymphocytic choriomeningitis.

2. *Allantoic sac inoculation* (see Fig. 10.10)—influenza, psittacosis and Newcastle disease. Technically much simpler than amniotic inoculation.
3. *Amniotic sac inoculation*—influenza, psittacosis, mumps and Newcastle disease. This route is the most sensitive for isolation of influenza and mumps from human sources. The detection of he-

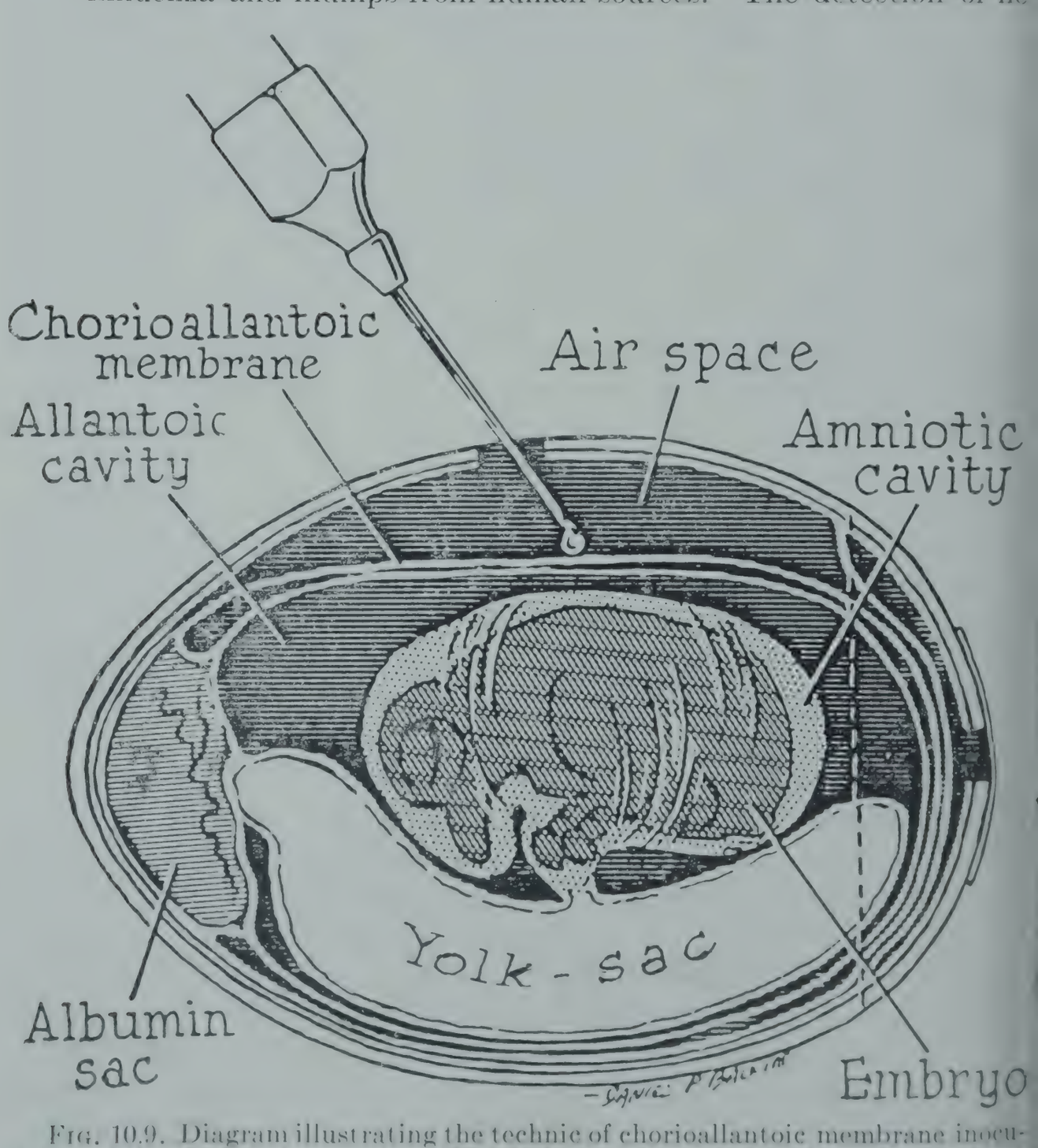


FIG. 10.9. Diagram illustrating the technic of chorioallantoic membrane inoculation of the chick embryo. An artificial air space is created as shown in the above diagram. The broken vertical line marks the inner boundary of the original air sac.

magglutinins for chick erythrocytes in the amniotic fluid is a presumptive finding for the presence of either influenza or mumps virus; an isolated virus is identified by the complement fixation or hemagglutination inhibition test. At least 3 or 4 blind passages

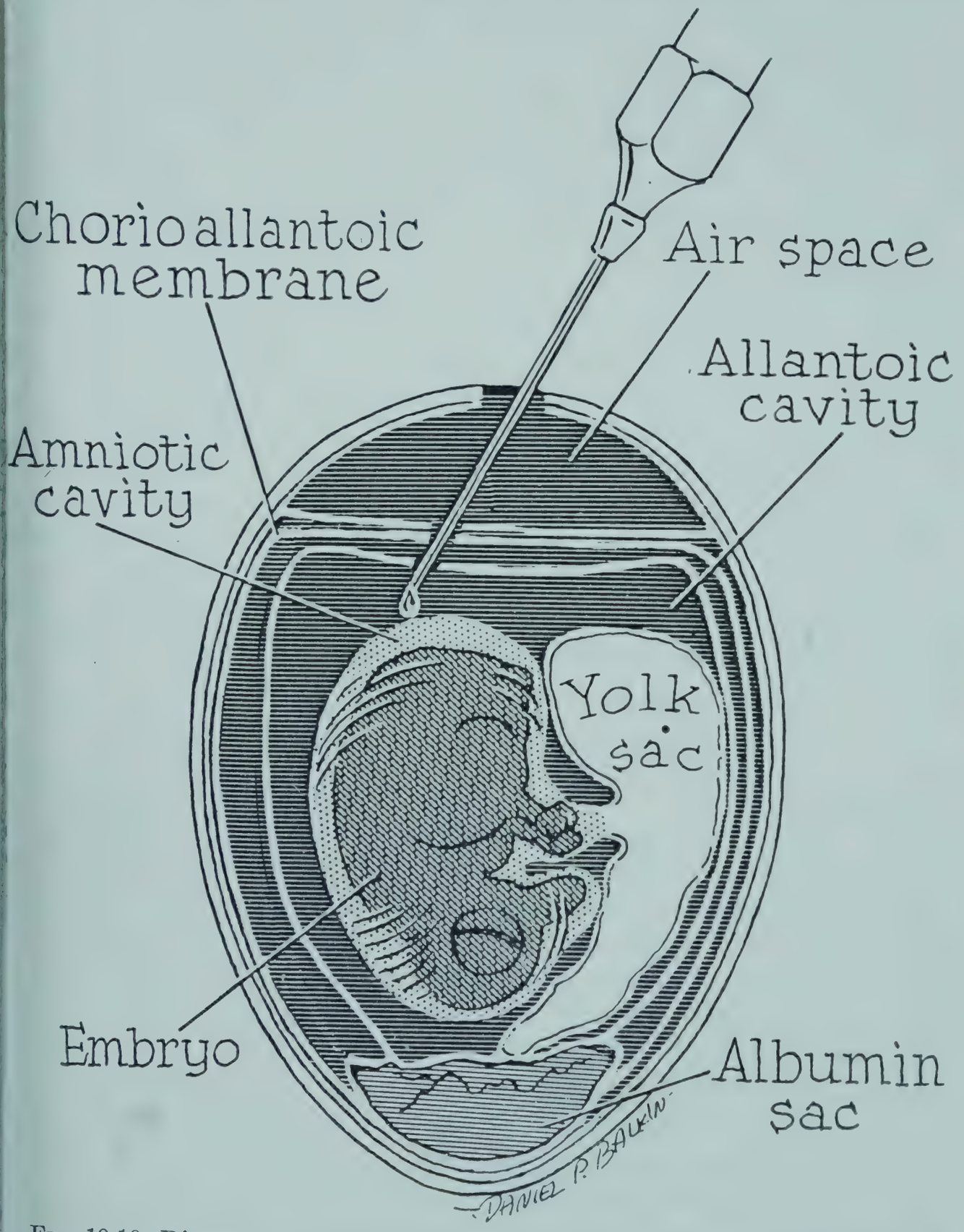


FIG. 10.10. Diagram illustrating the technic of allantoic sac inoculation of the chick embryo.

should be attempted using this technic of inoculation before regarding the results as negative.

4. *Yolk sac inoculation*—influenza, psittacosis-lymphogranuloma group, herpes simplex and mumps. Optimum multiplication of all the rickettsiae occurs in the yolk sac. Guinea pig inoculation is more sensitive for primary isolation. The usual technic is to establish the strain in a guinea pig and then transfer to the chick embryo by yolk sac inoculation. Cultivation of rickettsiae in the yolk sac of the developing chick embryo (Cox, 1938) is widely used at the present for the commercial manufacture of rickettsial vaccines and diagnostic antigens as well as for chemotherapy testing.

As in animal inoculation it is imperative that the inoculum be bacteriologically sterile or the embryo will die, frequently long before any appreciable effects of virus infection can be noted. Penicillin and streptomycin mixtures consisting of a final concentration of 100 units or micrograms of each per egg are generally effective for decontamination of the inoculum. Keep the inoculum in the refrigerator for two hours or at room temperature for a half hour prior to the injection. Inoculated embryos are usually incubated at 35 to 36°C. in a bacteriologic incubator.

Many technics for chick embryo inoculation have been described, but that advocated by Beveridge and Burnet (1946) or Goodpasture and Buddingh (1948) have given good results in my experience for diagnostic work. Their publications should be consulted for further details.

INTERPRETATION OF DIAGNOSTIC TESTS

A diagnosis of a viral or rickettsial infection should not be made on the basis of negative bacteriologic findings in a patient suffering from an acute febrile illness. Errors in the collection of material for culture or technical difficulties in the isolation of bacteria or other microorganisms may account for the failure. The same applies to making a diagnosis by lack of response to available antibiotic or chemotherapeutic agents. The improper drug may have been selected for use or inadequate quantities given. Likewise, the presence of a leukopenia does not necessarily imply a viral infection. Some viruses such as equine encephalomyelitis often produce a leucocytosis while bacterial infections, especially when severe, may be accompanied by a leukopenia. It cannot be overemphasized that the diagnosis must not be one of exclusion but should be made from positive epidemiologic, clinical or laboratory evidence of infection.

Animal or chick embryo inoculation is a research procedure and is not

practical for the routine laboratory diagnosis of viral or rickettsial diseases. Isolation studies are justified in outbreaks of public health importance of unknown etiology and in the diagnosis of fatal cases of suspected viral or rickettsial infections. When requesting this type of examination the physician should be willing to collect and ship specimens in accordance with the principles described in this chapter. It must be kept in mind that the chances of finding an infectious agent are greatly diminished if the specimen is not obtained immediately after onset of illness. Moreover the *failure* to isolate a viral or rickettsial agent cannot be interpreted as ruling out an infection of this category. This method of examination rarely offers any advantage in speed of diagnosis over serologic tests.

In all serologic tests a diagnosis should not be made from results with a single serum specimen. The positive result may be due to a past infection (anamnestic reaction) or previous vaccination. The importance of obtaining paired serum samples during the acute and convalescent phases of illness has been stressed repeatedly. Acute phase serum should be collected as soon as possible after onset of illness (preferably before the fifth day) and convalescent phase serums at least two and four weeks after onset. As a rule a negative or weakly positive reaction in the acute phase followed either by a positive reaction or a *fourfold* or more rise in antibody titer is diagnostic. Table 10.4 emphasizes the importance of taking serum specimens at various intervals during the course of a virus infection. Also the observation that neutralizing antibodies appear and decline independently of the complement fixing antibodies is readily seen in Table 10.4. Vaccination should be suspected whenever an antibody response occurs to both influenza A and B. As a rule, the infecting virus will produce the greatest rise in homologous antibody titer. Cross-reactions are apt to occur between St. Louis and Japanese B encephalitis since these viruses are antigenically related. Again the antibody response to the homologous virus will be the greater.

Complement fixation is recommended as the serologic test of choice for the routine diagnosis of many viral and rickettsial diseases by the hospital laboratory (Milzer, 1950; Harding *et al.*, 1954). Some laboratories may prefer to substitute the hemagglutination inhibition test for diagnosis of influenza and mumps infections. There is good agreement between these two types of tests as a diagnostic aid (Aikawa *et al.*, 1949). Lennette (1954), however, feels that the complement fixation test is preferred for the diagnosis of influenza and mumps because this test is relatively insensitive to virus strain differences and is little hampered by

the presence in human serum of non-specific substances which may interfere with the hemagglutination inhibition technic (Francis, Jr., 1947). Close liaison must be maintained between the clinician and the laboratory so that the proper group of diagnostic antigens will be selected for tests in a given case, depending on the clinical observations. For example, serums of a patient with atypical pneumonia are tested with the respiratory group of complement fixing antigens listed in Table 10.6.

The encephalitides group of antigens in Table 10.6 are employed for patients with encephalitis and the aseptic meningitis group including various leptospiras, (see Chap. 12) for those with virus meningitis. The rickettsial group are used for patients whose clinical condition is suggestive of infection with these organisms. When adequate antibiotic

TABLE 10.6

Classification of Diagnostic Viral and Rickettsial Complement Fixation Antigens for Clinical Use

A. Respiratory group
   1. Influenza A, A-prime, B and C
   2. Psittacosis-ornithosis
   3. Lymphocytic choriomeningitis*
   4. Q fever

B. Encephalitides group
   1. Western equine encephalomyelitis*
   2. Eastern equine encephalomyelitis*
   3. Lymphocytic choriomeningitis*
   4. St. Louis encephalitis*
   5. Mumps
   6. Jap. B encephalitis*

C. Aseptic meningitis group
   1. Lymphocytic choriomeningitis*
   2. Mumps
   3. Lymphogranuloma venereum†

D. Rickettsial group
   1. Rocky Mountain spotted fever
   2. Murine typhus
   3. Epidemic typhus
   4. Rickettsialpox
   5. Q fever

* Positive findings should be checked by neutralization tests.
† Positive findings should be checked by Frei intradermal test.

therapy is given early during the acute phase of a rickettsial infection, however, antibody response may be delayed or reduced to the extent that interpretation of the complement fixation test may be very difficult (Rivers, 1952). Tests for cold hemagglutinins and Streptococcus MG agglutinins must also be done routinely in cases of atypical pneumonia because these tests are of confirmatory value in diagnosis.

Hemagglutination inhibition is a widely used test for the diagnosis of influenza and mumps virus infections. Diagnosis depends upon the demonstration of at least a *fourfold* rise in antibody titer. The majority of normal human serums have influenza virus titers below 1:160, while titers of convalescent serums range from 1:320 to 1:10,240 or higher. In mumps the acute phase serums titer 1:40 or lower, and the convalescent serums from 1:40 to 1:640 or higher. There are a number of practical difficulties in the routine use and interpretation of the agglutination inhibition test. First, this technic is relatively strain specific for influenza virus so that from the diagnostic standpoint antibody rises may not be detected if the test antigen is markedly different from the strain causing the outbreak (Lennette, 1954). Secondly, the reliability of the agglutination inhibition test is affected by the presence of nonspecific inhibitors in human sera.

The hemagglutination inhibition test has been used in the past for the diagnosis of Newcastle disease in man. Recently, however, Jungherr et al. (1949) have shown that many mumps convalescent phase serums apparently also contain both antihemagglutinins and neutralizing antibodies for Newcastle virus. For this reason these workers expressed their belief that a diagnosis of Newcastle disease in man should only be made with caution, especially in the absence of virus isolation.

Cold hemagglutinin titers of 1:32 or more in convalescent phase serums of atypical pneumonia patients are considered suggestive. A fourfold or greater rise in titer is diagnostic. The final titer is generally proportional to the severity of illness. Cold agglutinins usually appear at the end of the first week of illness or during the second week, reaching a peak from the middle of the second to the middle of the fourth week, and disappear or decrease in titer from the fourth to the sixth week. For this reason, a decrease in titer later in convalescence is significant.

Titers above 1:128 are seldom encountered except in atypical pneumonia or certain hemolytic anemias. It must be emphasized that the presence of cold agglutinins is not pathognomonic for atypical pneumonia because these antibodies are detected in only about 50 per cent of patients (Horsfall, 1948). Cold agglutinins have been described in other

diseases such as blackwater fever, trypanosomiasis, mumps orchitis, hemolytic anemias, certain hepatic diseases, peripheral vascular disease, etc. These agglutinins generally in low titer may also occur in a variety of common respiratory diseases including bronchopneumonia. They are usually absent in the serum of psittacosis and Q fever patients.

Streptococcus MG agglutinins and cold hemagglutinins are unrelated antibodies and may occur simultaneously or independently in cases of atypical pneumonia. Streptococcus MG agglutinins are detectable by the second or third week after onset and reach a peak during the fourth and fifth weeks. As in the case of cold agglutinins, the titer of streptococcus MG antibodies can often be correlated with the severity of illness. Streptococcus MG titers of 1:20 or more have been reported in 4 to 50 per cent of atypical pneumonia outbreaks (Horsfall, 1947). The demonstration of a fourfold or greater rise in titer during convalescence is considered significant. Low titers without the rise are encountered in normal persons, various acute respiratory diseases and in patient with certain localized streptococcal infections.

The fact that such known viral and rickettsial diseases as psittacosis, influenza, lymphocytic choriomeningitis and Q fever may closely simulate atypical pneumonia serves to emphasize the diagnostic value of conducting serologic tests for patients with infections of the respiratory group. Furthermore, the full benefits of antibiotic and chemotherapy are more apt to be achieved if the exact etiologic basis of the disease is known (see Chapter 9, section on clinical use of sulfa drugs and antibiotics). It must also be kept in mind that pulmonary tuberculosis, bacterial pneumonias, pulmonary mycoses or toxoplasmosis may present clinical manifestations which are indistinguishable from those of atypical pneumonia. In fact, Kunstadter *et al.* (1949) have reported a proven, nonfatal case of primary histoplasmosis in a patient with the clinical observations of atypical pneumonia.

The value of conducting routine serologic tests in infections caused by the encephalitides group of viruses is evident because these infections usually cannot be differentiated by clinical or pathologic observations. Two or more neurotropic viruses may be endemic in the same region, i.e., those of St. Louis encephalitis and Western equine encephalomyelitis in certain of the Western states. Laboratory aid is also essential for the diagnosis of lymphocytic choriomeningitis because of the protean clinical manifestations that this virus produces and the fact that this infection is highly sporadic in man (Armstrong, 1942; Milzer, 1943). Nine proven cases which the author studied were clinically divisible as follows: (1) en-

cephalomyelitic type, (2) meningeal type and (3) and "influenzal" type without neurologic signs.

Positive complement fixation with the encephalitis group of antigens should be checked by the neutralization test (Milzer, 1950; Harding *et al.*, 1954). Perhaps it would be advisable for laboratories with facilities only for routine tests to send serums positive for viruses of the encephalitides group to the reference virology laboratories for tests in verification of the diagnosis. In my experience complement fixing titers in the neurotropic group rarely exceed 1:40, while titers in excess of 1:160 are not unusual in the rickettsia and psittacosis groups.

There is increasing evidence that the mumps virus is a common cause of meningoencephalitis without salivary gland involvement and nonparalytic poliomyelitis (Kilham *et al.*, 1949; Milzer, 1950). The complement fixation test is especially useful in establishing the diagnosis in these cases. The demonstration of a fourfold increase in antibody titer during the course of the illness is regarded as conclusive evidence of a mumps infection. Enders (1948) points out that serum specimens in such cases are often taken too late to permit the demonstration of a rise in antibody titer. He regards the finding of a titer exceeding 1:64 in a single specimen of serum as presumptive evidence of recent mumps infection. The serums of normal persons rarely reach this titer, except if infected within the preceding year. The mumps skin test is of value in determining an individual's immune status but should not be used for diagnosis. First, it rarely becomes positive until several weeks have elapsed after onset of mumps. Secondly, it stimulates the production of complement fixing antibodies or increases the titer of those already present, producing false positive reactions.

The complement fixation test can not be used to differentiate the psittacosis-lymphogranuloma group of viruses because of cross fixation (see Chapter 18, section on lymphogranuloma venereum). Other members of this group which infect man include the ornithosis and human pneumonitis viruses (S.F., Louisiana and Illinois strains). Identification of an individual virus of this group is a research procedure. Complement fixation titers in a dilution of 1:32 or greater are considered highly suggestive of infection by this group, but a fourfold rise in antibody titer is considered significant when the early and late serum samples are tested simultaneously. The intradermal Frei test with lygranum antigen (see Chapter 18) also shows cross reactions between members of this group but is regarded as less sensitive than the complement fixation test (Rake *et al.*, 1941). If both tests are positive, the diagnosis must

be evaluated in the light of epidemiologic and clinical findings. Like the mumps skin test, the Frei antigen stimulates the formation of complement fixation antibodies and should not be done before completing the serologic tests.

As in many of the viral infections, the complement fixation test is being employed for the routine diagnosis of the rickettsial infections with increasing frequency. Specific complement fixing antibodies are present in the convalescent serums of patients infected with the spotted fever group, typhus group, scrub typhus group and Q fever. Laboratory diagnosis depends on demonstrating a significant rise in specific antibodies during convalescence as in other serologic tests (Smadel, 1948a). Ordinarily the complement fixation test is helpful in differentiating epidemic typhus, murine typhus and Rocky Mountain spotted fever when washed rickettsial antigens are used, while the Weil Felix reaction (see Chapter 11) is of little or no value for specific diagnosis of these rickettsial diseases. Furthermore, positive Weil Felix reactions have been reported with serums from cases of nonrickettsial origin (Wertman, 1948). The Weil Felix test is negative in Q fever and rickettsialpox. Schubert *et al.* (1954) have recently emphasized the need for standardizing the technic of the Weil-Felix test and have indicated that the latter is still a useful diagnostic tool, especially since antibiotic therapy may interfere with or prevent the formation of complement fixing antibodies apparently without affecting the serologic responses as measured by the Weil-Felix test. Cross complement fixations occur between Rocky Mountain spotted fever and rickettsialpox, but the highest titer is usually obtained with the homologous antigen if washed rickettsiae are used in the test.

Individuals vaccinated with epidemic typhus vaccine generally show complement fixing titers ranging from 1:4 to 1:32 with the epidemic antigen. According to Zarafonetis (1945) such vaccinated individuals do not develop increased titers when suffering from a febrile illness of nontyphus etiology. Furthermore, when such vaccinated persons are subsequently infected with either epidemic or murine typhus they develop a prompt rise in both homologous and heterologous antibodies, making a specific diagnosis from serologic data impossible (Smadel, 1948b).

All of the complement fixing antigen groups listed in Table 10.6 may profitably be used to test the serums of patients with fever of unknown origin. Tests for heterophile, cold and streptococcus MG agglutinins should be included. It is important to rule out bacterial, mycotic or parasitic infections in these patients. The finding that approximately

14 per cent of serologic tests exclusive of heterophile agglutination were positive in the author's series of 85 patients with fever of unknown origin serves to emphasize the value of conducting routine diagnostic tests for viral and rickettsial diseases (Milzer, 1950). These results compare favorably with recent reports by Adair *et al.*, 1953 and Harding *et al.*, 1954. It should be remembered, however, that other viral diseases exist for which no laboratory tests are available at present such as serum jaundice or infectious hepatitis. Undoubtedly, many infections of unknown etiology are caused by viral agents which remain undiscovered. This is emphasized by the fact that several new viruses (Robbins *et al.*, 1954) have been isolated recently from patients that are pathogenic only for tissue culture of human or monkey origin, while standard laboratory animal and chick embryo inoculation with these agents have given negative results. Finally, the present discussion has been largely limited to the more common viral and rickettsial diseases known to exist in the United States for which diagnostic serologic tests are available. At least 60 different viruses and rickettsiae are known to cause disease in man (Rivers, 1952).

Another group of viruses which merit some discussion are the Coxsackie viruses (Dalldorf, 1948). These viruses are pathogenic for only suckling mice and hamsters and have been isolated from the feces and throat washings of patients with paralytic and nonparalytic poliomyelitis. Strains of Coxsackie virus vary in the site and severity of lesions that they produce and are separated into Type A and B on the basis of their histopathological effect in infant mice (Huebner *et al.*, 1952). Type A causes widespread myositis in skeletal musculature, while Type B produces focal muscle lesions with accompanying degenerative effects in the brain, liver, pancreas and fat pads. Strains of Type A are chiefly associated with herpangina, while Type B have usually been found responsible for epidemic pleurodynia (Bornholm disease or epidemic myalgia) and aseptic meningitis. The Coxsackie group of viruses are serologically heterogenous; Type A is composed of 11 types and Type B of 5 types.

Diagnosis is made by the isolation of virus in suckling mice from the feces, throat swabs or pharyngeal washings followed by immunological typing of the isolated strain. Confirmation of infection is established when a significant rise in antibody titer is demonstrated against the isolated strain in neutralization tests performed on acute and convalescent phase sera in suckling mice. The complement fixation test cannot be recommended at this time for demonstrating infection because of the

development of heterotypic antibody elevations (Beeman *et al.*, 1952). Isolation of the Coxsackie virus, its identification, and the demonstration of a rising titer of antibodies are still too laborious for routine use.

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CHAPTER 11

IMMUNOLOGIC TESTS

By Ralph McBurney

GENERAL CONSIDERATIONS

Immunological tests are based upon either antigen-antibody reactions or hypersensitivity, and are extremely important and useful as diagnostic laboratory or clinical procedures. The presence of sensitizing antibodies in a given serum may be demonstrated by agglutin, precipitin and complement fixation tests, which are in vitro in nature. In vivo antigen-antibody reactions are represented by the Dick test for susceptibility to scarlet fever, the Schick test for diphtheria, the Schultz-Charlton test for diagnosis and differentiation of scarlet fever from German measles and certain drug idiosyncrasies.

Hypersensitivity tests are, for the most part, clinical and in vivo in nature. Classical examples are tests for hypersensitivity to foods, plant pollens, fungus spores, animal dander, feathers, house dust, certain drugs, horse serum, the tuberculin, mallein and numerous other tests.

Many of these do not involve laboratory procedures and therefore find no place in texts of this nature but may be found in detail in standard texts of bacteriology, immunology and clinical medicine.

Past concepts of antigen-antibody reactions were based upon Ehrlich's theory of receptors, which attempted to portray reactions of this type by graphic illustration of hypothetical reactions. Jordan and Burrows stated that such was never intended to be other than an imaginary picture constructed to convey a concept difficult to put in words. Modern views of the mechanisms of these reactions, which are partly physical and partly chemical in nature, assign them to the realm of colloidal chemistry. Modern concepts are given under each type of reaction considered.

Bacteria and other antigens gaining entrance into the animal body through disease or immunization processes stimulate the production of various types of antibodies which tend to destroy offending microorganisms outright, limit their spread, or neutralize toxins they may elaborate. These antibodies are specific in their reactions with the protein, lipoid and complex carbohydrates that are constituents of the particular antigen giving rise to their formation. The demonstration of specific antitoxins, opsonins, agglutinins, precipitins, or complement-

binding antibodies constitutes definite evidence of their presence in the animal body. Agglutination tests have long been used as diagnostic aids in these antigen-antibody reactions. They should not displace procedures for the isolation and identification of the specific etiologic agent.

Antibodies are found mostly in blood and lymph. They are probably formed by cells of the reticuloendothelial system and are globulins modified by the animal body in response to stimulation of the antigen giving rise to their formation and for which they are specific. There is great variation in the quantity of antibodies produced in response to the stimulus from various antigens.

Many diseases may be diagnosed by taking advantage of the specificity of reactions obtained when, under proper conditions, (1) known antigens are mixed with serum of the patient containing unknown antibodies, or (2) known diagnostic serum containing specific antibody is mixed with unidentified microorganisms. Thus, serological procedure may be employed in the diagnosis of disease by one or both of two methods: (1) the patient's serum may be mixed with known organisms; (2) organisms isolated from the patient may be mixed with known diagnostic sera. This is especially applicable to agglutination tests.

Precipitin tests are likewise based upon antigen-antibody reactions. However, the antigen, if bacterial in origin, is a clear filtrate containing the metabolic products of specific microorganisms; if of animal origin, a saline extract of cells or tissue.

In agglutination tests microorganisms or erythrocytes are clumped through the action of specific antibodies. In precipitin tests flocculant precipitates are formed through the interaction of two clear fluids representing antigen and antibody respectively.

Precipitin tests, with exceptions, are not routine in clinical laboratories but find most useful application in forensic medicine for identifying animal species from their tissues or blood stains and for determining the feeding habits of certain mosquitoes by identifying the type of animal blood in the stomachs of these insects.

Precipitin tests as applied to bacteria are important and useful as illustrated in the Lancefield method of typing beta hemolytic streptococci. The unknown organism is extracted with acid and heat, resulting in a clear solution. This is layered over small amounts of rabbit sera, each containing specific antibodies produced by inoculating rabbits with known types of organisms—the unknown type of organism being indicated by the serum which produces a precipitate.

Complement fixation tests depend upon not only antigen-antibody response but also upon a third component complement—a thermolabile constituent of normal animal sera. As applied to the detection of syphilis the antigen (a cardio-lipin extract of beef heart) is non specific, acting in a specific manner. This is similarly true of the detection of certain parasitic diseases such as hydatid cysts in man, formed from the dog tapeworm, *Taenia echinococcus*, in which case the antigen is group specific. As applied to forensic medicine, specific antigens in the form of soluble extractives of cells or tissues are employed.

Other uses are discussed in this chapter under Complement Fixation Tests.

Detailed discussions respecting this test for syphilis will be found in Chapter 19, Syphilis Serology.

The role of opsonins as defensive mechanisms in the body, through sensitization of bacteria for englulfment by the polymorphonuclear leukocytes, has been well demonstrated in vitro by laboratory procedures. While electrolytes are accessory substances in agglutination and precipitation reactions, complement in complement fixation, bacteriocidal and bacteriolytic reactions, leukocytes are accessory substances in opsonocytophagic reactions. Formerly the opsonocytophagic test of Huddleson was recommened as a routine in suspected brucellosis, but is no longer considered an aid in the diagnosis of this condition. As far back as 1940 Wise, in a study of the evaluation of this test, concluded that it is not diagnostic of active *Brucella* infection and may be negative even when active infection exists. Castaneda, Tovar and Velez, in 1942, through a comparative study of various diagnostic tests in brucellosis occurring in Mexico, stated that this test was not always demonstrable in infection of this type. In 1952 Spink, McCullough, Hutchings and Mingle, in their report of the National Research Council Committee on the public health aspects of brucellosis, stated that this test is complicated, and to be of value must be precisely controlled; that slight variations in the test culture may render the test worthless; that it must be performed shortly after blood is drawn; that it is not a practical test for routine use; that the information which it provides is of slight value in diagnosis or management of cases; and that it is not recommended for use.

AGGLUTINATION TESTS

As a laboratory diagnostic aid, agglutination tests have a proven value, not only in diseases of bacterial origin but in many others. Their

usefulness in bacterial infections is well recognized in the diagnosis of *Salmonella* infections such as typhoid and paratyphoid fevers, brucellosis, tularemia, bubonic plague, glanders, pertussis, determination of *Neisseria meningitidis* groups in epidemiological surveys, detection of the presence of *Streptococcus MG* in primary atypical pneumonia and the identification of Salmonella and Shigella cultures, etc.,

The Weil-Felix agglutination test in certain rickettsial diseases is well known. A practical and specific slide agglutination test for *Trypanosoma cruzi* infections in man was developed by Senekjie (1943) and for *Leishmania tropica* infections by Senekjie and Lewis (1944). Howe (1943) developed a group specific agglutination reaction for the diagnosis of Carrion's disease (bartonellosis).

Agglutination tests in which sheep erythrocytes are employed as a non-specific (heterophile) antigen are used as an aid in differentiating infectious mononucleosis and monocytic leukemia. Agglutination tests are also used in hemagglutination of human erythrocytes in determining the blood groups and the Rh factor. Our knowledge of cold hemagglutinins, in which mixtures of serum and erythrocytes of the same individual results in clumping of the erythrocytes in the cold from 0 to 5°C., finds application in primary atypical pneumonia, in trypanosomiasis, (human and animal), sometimes in mumps, in various kinds of hemolytic anemias and in Raynaud's phenomenon in association with gangrene following exposure to cold.

Mechanism of Agglutination Reactions

Smooth strains of microorganisms tend to remain dispersed in a suspension of 0.85 to 0.9 per cent solution of sodium chloride, resulting in an even suspension, since they carry sufficient negative charge to overcome the slight cohesive effect they exert upon each other.

At the pH of body fluids immune serum also carries a negative charge which is definitely weaker than that on the bacterial cell.

When suspensions of living or killed bacteria or other cells such as erythrocytes, in optimal saline concentration, are added in proper proportion to specific antisera, the colloidal antibodies are adsorbed on the surface of the antigen which is thereby sensitized. As a result the charge on the organisms is diluted to a point whereby it is not able to overcome the cohesive effect which the organisms exert upon one another, thus the relatively discharged organisms tend to agglutinate. The combination of the antibody with bacteria also increases the cohesive force between them. The rate of agglutination is varied by the

concentration of salts, quantity of agglutinin, proximity of the bacteria to one another, temperature of reaction and cohesion.

The reaction therefore takes place in two stages:

1. Adsorption of antibody (agglutinin) on the surface of antigen which is thereby sensitized.
2. Agglutination of this salt-sensitive antigen-antibody combination by the electrolyte.

Boyd, referring to the work of Pijper, in which motion pictures taken with the aid of sunlight illumination and the electron microscope reveal that at least three kinds of bacterial agglutinins seem to be possible with certain flagellated bacteria, states:

1. Flagella (H) agglutination seems to be the result of the deposition of agglutinin on the flagella in the form of a film of antibody molecules radially disposed (standing on end). The flagella become immobilized and later entangled mechanically, resulting in agglutination.
2. In somatic (O) agglutination the antibody appears to combine with the body of the cell; bacteria thus "sensitized" no longer repel but become attracted to each other.
3. In "Vi" agglutination Pijper believes that three factors operate:
   a. An increasing paresis of motile organisms,
   b. Continued repulsion between bacilli,
   c. "Stickiness" of the surface of the bacteria—resulting in their sticking together when they collide.

These observations by Pijper, using *Salmonella typhosa*, are illustrated in Figures 11.1–11.4.

Types of Reactions

Two distinct types of reaction are in use: microscopic and macroscopic. The microscopic test is particularly applicable to living, motile bacteria, such as those of the *Salmonella* causing typhoid or paratyphoid fevers and other types of infection. If facilities for keeping cultures are available, this type of reaction may be performed with some saving of time. Living organisms are not killed nor is their virulence altered by agglutination. Therefore extreme care should be observed in handling. As a matter of safety, the macroscopic test is usually applied to dead or non-motile organisms, the agglutinability of which is not altered by killing. This test is a much better index of the quantity of antibody present. Bacterial suspensions used for the macroscopic test may be

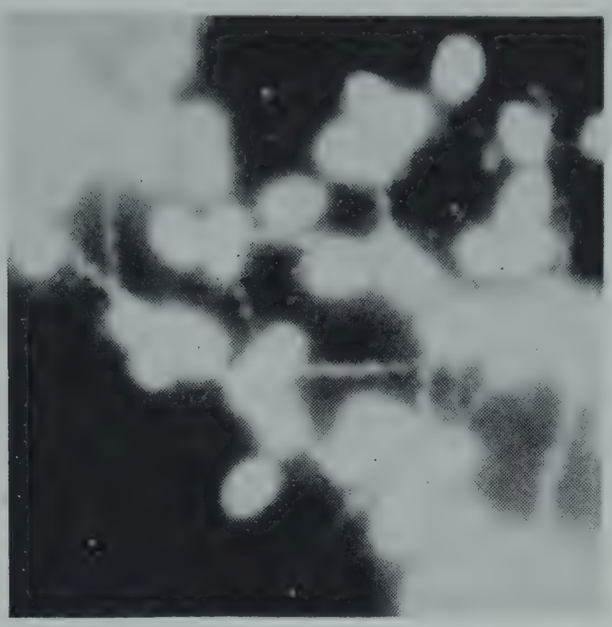


FIG. 11.1. H agglutination.



FIG. 11.2. Early stage of O agglutination.

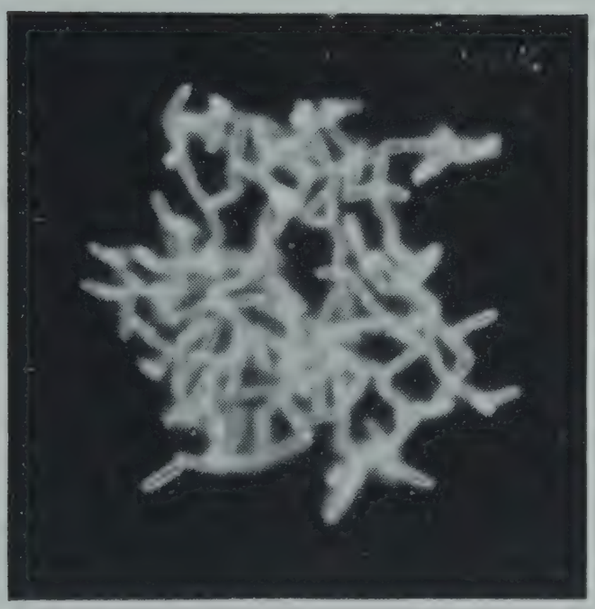


FIG. 11.3. Late stage of O agglutination.



FIG. 11.4. Vi agglutination.

FIGS. 11.1–11.4. Agglutination reactions of *S. typhosa* as observed in darkfield. Reproduced from Smith and Conant: Textbook of Bacteriology, 10th Ed., p. 180. Appleton-Century-Crofts, Inc., New York, 1952.

prepared and kept in a refrigerator for several months. They may also be obtained from biological supply houses.

Pure cultures of organisms desired for antigen may be obtained from the American Type Culture Collection, Georgetown University School of Medicine, 3900 Reservoir Road, N.W., Washington, D. C. Specific antigens and antisera may be obtained from A. S. Aloe Company, 5655 Kingsbury, St. Louis 12, Mo., and from Lederle Laboratories, Inc., 30 Rockefeller Plaza, New York 20, New York.

The macroscopic slide or "spot" agglutination test is a time saver and

may be applied to suspicious isolated plate colonies. It may also be used to determinine the presence or absence of specific antibodies. A good example of this is the Huddleson rapid slide agglutination test for brucellosis in which a constant volume of standard *Brucella* antigen is added to varying dilutions of serum from a suspected case of brucellosis. The test is fully described elsewhere in this chapter.

Either type of agglutinating reaction may be used to determine the presence of circulating antibodies in body fluids, especially serum, or for determining the titer of diagnostic serum employing a saline emulsion of the homologous organism. For example, in a person suspected of typhoid fever, his serum recovered from blood taken after 7 to 10 days will agglutinate a known strain of *S. typhosa*. On the other hand, an organism having the morphology and staining characteristic of *S. typhosa* isolated from a blood culture taken in the early stages (3 to 5 days following symptoms) may, when used as antigen with a known antityphoid agglutinating serum, be agglutinated by the homologous antibodies in the known serum. Thus, agglutination tests may be used (1) to determine the presence of an unknown antibody using a known antigen; and (2) to determine the presence of an unknown antigen using a known antibody.

While the agglutination test is the simplest to perform and has the widest range of usefulness of all serologic technics, there are many points to be considered, and variables will be encountered which may render the test useless if they are not recognized.

Agglutination Titer

On setting up agglutination tests it is customary to prepare serial dilutions of serum on slides or in serologic tubes to which are added constant amounts of a saline suspension of antigen. After thorough mixing and incubation for an appropriate interval, usually at 37°C., they are examined for the presence of flocculation of the antigen. The tube containing the highest serum dilution in which this occurs represents the *titer* or relative antibody concentration of the serum. Thus a serum showing agglutination in a dilution of 1:10,000 but not in 1:15,000 is said to have an agglutination titer of 1:10,000.

Raffel prefers to consider *titer* as an expression of the number of antibody units per unit volume of undiluted serum, unit volume being 1 ml. For example, if the last tube showing a positive reaction contains 1 ml. of a 1:1000 dilution of serum, the titer of the serum is 1000 units of antibody per ml. He states that expressing titer as a function of dilu-

tion without reference to unit volume, as is often done by different laboratories, makes comparison impossible unless sufficient data are given to permit conversion to a unit volume basis.

Agglutination titers vary to a degree depending upon the density of the antigen suspension: light suspensions showing a faint turbidity to the eye give higher titers than dense suspensions.

Zones of Agglutination

Three zones may be observed when an agglutinating serum containing constant amounts of homologous antigen is sufficiently diluted.

I. A point of dilution toward the right beyond which no agglutination occurs in successive tubes. This is due to lack of sufficient serum antibody to cause agglutination of the suspended cells, and is defined as the "*post zone.*"

II. On the other hand at the left where serum antibody concentrations in the first few tubes are highest (1:100 or less) no agglutination may occur. This is the zone of inhibition, and is called the "*prezone,*" "*prozone,*" or "*proagglutinoid zone.*"

III. Between these zones, where optimal concentration of antiserum is present in which agglutination occurs, is the "*zone of agglutination.*"

A diagrammatic representation of these zones, after Boyd, is depicted in Figure 11.5.

An understanding of the zone of inhibition is of practical importance because, if antisera are not diluted sufficiently, tests otherwise positive may be incorrectly reported negative. This is a variable phenomenon, some antisera exerting a greater inhibitory action than others. Certain

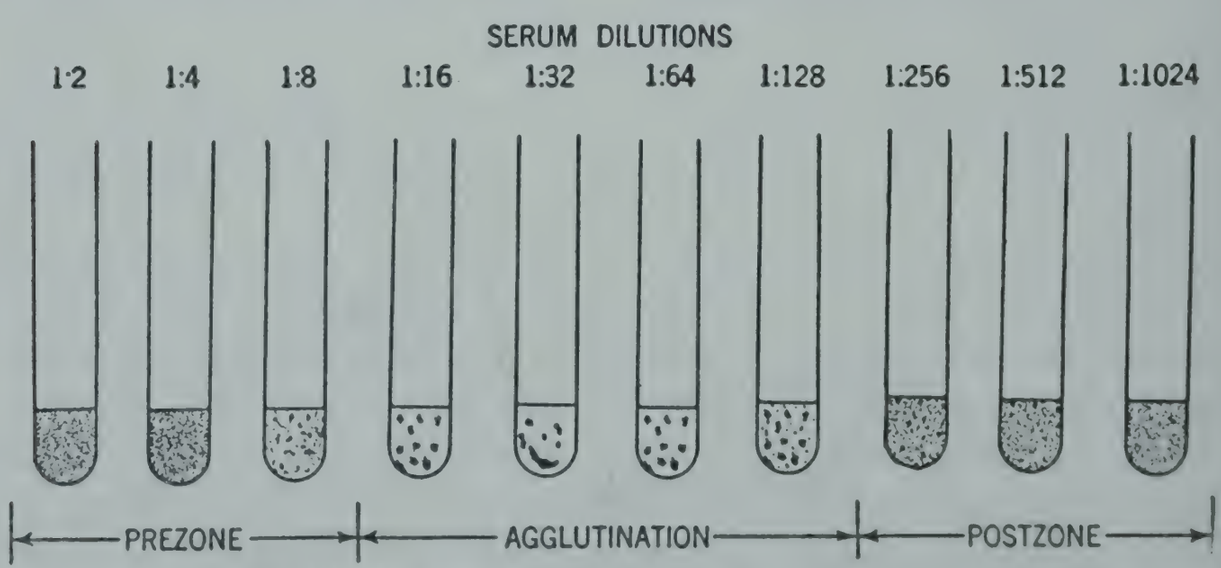


FIG. 11.5. Serum dilutions. Diagrammatic representation of zones in agglutination. From: Fundamentals of Immunology, by William C. Boyd, 2nd Ed. Interscience Publishers, Inc., New York, 1947.

antigen suspensions such as *Brucella* and *Pasteurella tularense* produce the same effect.

There have been a number of theories to explain this phenomenon but none is entirely satisfactory. Ehrlich held that aging, heat and slight acidity caused the formation of "agglutinoids," having a greater affinity for the antigen than agglutinins. This action was thought to be overcome by dilution. The more modern concept, as stated by Smith and Conant, holds that the albumin fraction of the serum interferes with the reaction between antigen and antibody globulin—either by nonspecific absorption of the albumin on the antigen, thereby blocking its sensitization by antibody, or by inhibiting the second stage of agglutination through secondary absorption on the surface of sensitized cells, thus preventing their aggregation.

Cross Agglutination

The antigenic pattern of bacteria is not always simple. A cell may consist of cytoplasmic (somatic) flagella and capsular antigens. The somatic and flagella antigens of closely related species within the same genera may contain a number of common antigens. This is characteristic of the *Salmonella*, while the same may be said of the somatic antigens of closely related non-flagellated species such as the *Shigella*.

Differences in the polysaccharidal capsular antigens are well illustrated in the *Pneumococci*, formerly of clinical value in typing the organisms for specific serum therapy.

These antigen relationships are not only characteristic of closely related species but unrelated ones may likewise possess common antigens. Thus type B. *Klebsiella pneumoniae* (a gram-negative bacillus) and type II *Diplococcus pneumoniae* (a gram-positive diplococcus) possess a common capsular polysaccharide.

When persons or animals have been infected with or immunized to an organism possessing antigens common to other organisms, their sera will agglutinate not only a suspension of the homologous organisms but also closely related ones containing similar antigens. This gives rise to what is termed cross agglutination and may usually be overcome in routine laboratory determinations by high serum dilutions, since titers are higher toward homologous organisms than hetrologous ones.

Cross reactions are characteristic of the *Salmonella*, *Shigella* and *Brucella* groups, each genera sharing antigens common to its group. Cross reactions between *Brucella* and *Pasteurella tularensis* are said to be common, but in the experience of Smith and Conant have been rarely

encountered. Distinction between these and other closely related species may best be made by agglutinin absorbtion tests. These antigenic relationships and the principles of absorption reactions are illustrated below.

Assume two closely related organisms of the Shigella group containing antigens giving rise to agglutinating antibodies indicated below:

	Antigens		Antibodies
Organism I	A | B | C | D	⟶	a | b | c | d
Organism II	C | D | E | F	⟶	c | d | e | f

If the antiserum of I, containing antibodies a, b, c, d, is absorbed by its homologous species containing antigens A, B, C, D, no antibodies will be found upon centrifuging and subsequent testing of the supernate. Similarly the same will hold true for organism II. However, if the antiserum of organism I, containing antibodies a, b, c, d, is absorbed by organism II, containing antigens C, D, E, F, only antibodies c and d will be absorbed leaving a and b unabsorbed with antigens E and F remaining.

Thus:

	Antigens		Antibodies
Organism II	C | D | E | F	+ Organism I	a | b | c | d
Organism II	| | E | F	+ Organism I	a | b | |

If, following centrifulization of this mixture, organism I containing antigens A, B, C, D, is added to a part of the supernate containing antibodies a, b, agglutination results. However if organism II, containing antigens C, D, E, F, is added to a part of the supernate containing only antibodies a, b no agglutination results, proving the organism in question to be I, as illustrated:

	Antigens		Antibodies	
Organism I	A | B | C | D	+ Organism I	a | b | |	= agglutination
Organism II	C | D | E | F	+ Organism I	a | b | |	= no agglutination

H and O Agglutinins

The possession of flagella (H) and somatic (O) antigens by certain bacteria and the respective agglutinating antibodies to which they give rise, is of extreme diagnostic importance—especially with respect to the *Salmonella* which possess both H and O antigens. Since H antigens are species specific and O antigens group specific, with respect to the antibodies they produce, it furnishes a means for identification and classification of the *Salmonella* species. This knowledge also makes possible a laboratory aid for distinguishing between agglutinating antibodies formed as a result of past or present infection, response to artificial immunization or nonspecific ("anamnestic") stimulation, through infection by an unrelated organism, of antibodies previously formed. This reaction may occur even six to nine years or more following immunization.

H and O agglutinating reactions are of particular clinical importance in distinguishing between antibodies formed as a result of infection and those in response to previous artificial immunization as in typhoid and paratyphoid fevers and to a certain extent in the detection of typhoid carriers; however the Vi agglutination test is much more reliable. H and O antigen suspensions are best made from pure smooth motile strains, the H with formalin, the O with alcohol which destroys the flagellar component. Their preparation and interpretation of reactions will be found under procedures and interpretation.

Normal Bacterial Agglutinins

Low titer antibodies for certain organisms may normally be found in the sera of some individuals and are probably formed in response to stimulation through contact with such organisms in sub-clinical infections or under normal conditions. It is conceivable that the bacterial flora of the nasopharynx and colon may afford opportunity for their formation. Because of their low titer (1:5–1:40) their presence in sera does not interfere with agglutination reactions.

Vi Antigens and Antibodies

Occasionally freshly isolated strains of *Salmonella typhosa*, particularly those isolated from the blood, in addition to H and O antigens possess a third one known as Vi antigen, which is exterior to the periphery of the O antigen, thus protecting the organism from the agglutinating action of anti-O serum. Vi antigen is also characteristic of *Salmonella hirschfeldii* (paratyphi C), and *ballerup*. Therefore such cultures may not be

agglutinated by their homologous antisera on primary isolation, but may be agglutinated following daily sub-culturing in nutrient broth for a period of four to five days, which destroys Vi antigen. Vi antigen may be destroyed by heating saline suspensions for one hour at 60°C., which does not destroy O antigens.

According to Peluffo (1941) Vi antigen may also be destroyed by heating saline suspensions containing 0.5 per cent phenol for 15 minutes or saline suspensions saturated with chloroform for 4 minutes at 50°C. This does not impair the activity of O antigen. Elimination of Vi antigen therefore makes possible union between O antigen and antibody resulting in agglutination.

Eliot (1940) found that over 95 per cent of chronic typhoid carriers contained Vi agglutinating antibodies. This and the work of Bhatnagar (1938) and of others has shown that the demonstration of Vi agglutinins in the blood serum of individuals is the most reliable method of typhoid carrier detection. Further consideration of this will be found under procedures.

Patient's Serum. In cases where the patient's serum is to be examined for the presence of agglutinating antibodies it is preferable to withdraw 3 to 5 ml. of blood by venepuncture into a sterile serological tube, or by employing any of the automatic vacuum type tubes such as the Keidel or others of this type. This procedure at least furnishes sufficient quantity for performing macroscopic tests for *Salmonella*, *Brucella*, *Rickettsiae* and syphilitic infections. Another advantage is the possible routine culturing of blood clots. A clot which is formed following withdrawal of blood should be detached from the walls of the container. Such specimens remain suitable for use for several days if kept in the refrigerator. If agglutination tests are to be performed right away it is not necessary to receive them in sterile tubes. All tubes, whether sterile or otherwise, should be chemically cleaned.

Bacterial Suspensions (Antigens). Bacteria grown in the laboratory for some length of time may dissociate into several types of colony growth, markedly deviating in motility and capacity for agglutination from the parent type present in disease. The use of smooth (S. type) strains is essential where this phase is considered the "normal" form of the organism since rough (R type) strains do not form a homogeneous emulsion in saline, agglutinate spontaneously and are poor antigens. However, exceptions are encountered, for example, in the anthrax bacillus and hemolytic streptococci where the finely granular (R types) are the virulent "normal" forms.

Detection of Rough Strains

Pampana macroscopic slide agglutination test may be used to detect roughness in the antigenic composition of organisms. The reagent consists of a 1:500 solution of trypaflavine (acraflavine) in normal saline.

Procedure.

1. Put a drop of the solution on a clean slide.
2. Close to the drop, but not in the drop, place a minute fraction of a loopful of the bacterial colony to be examined.
3. Flame, cool and moisten the loop with a drop of physiologic saline.
4. Emulsify the bacterial material with the moistened loop and finally mix with the drop of trypaflavine.

If the colony is rough, agglutination occurs immediately or within a few seconds. The reaction is more easily read if the surface of the slide is illuminated by oblique light against a dark background.

Care must be taken to gradually mix the bacterial emulsion and the whole droplet of trypaflavine, otherwise pseudo-agglutination may occur.

Preparation of Bacterial Antigens

It has been previously stated (p. 592) where various antigens and antisera may be obtained commercially; however, many workers find it more convenient and satisfactory to prepare their own.

Because of possible accidental infection it is not customary to use living antigens except in the performance of the slide agglutination test for typhoid and paratyphoid fevers, in tube tests for rickettsial infections using Proteus OX strains, and in leptospiral infections where a mixture of active motile *Leptospira icterohemorrhagiae* and *L. canicola* is employed.

A pure culture is essential in the preparation of any bacterial antigen. This consists of plating on a suitable medium, picking suspected colonies, determination of morphological, staining and biochemical characteristics, serological testing against a known specific positive antiserum of suitable titer, negative serum and saline controls.

Salmonella. Maintain stock cultures of a S-motile strain in nutrient bouillon or on agar slants kept at room temperature. For the microscopic test, a culture should be incubated at 37°C. for 18 to 24 hours to give a maximum concentration of living, motile organisms.

The modern macroscopic test for *Salmonella* agglutinins requires familiarity with the complex antigenic structure. Weil and Felix designated the flagellar antigens as H antigens and the somatic antigens as O

antigens. Ordinarily, H antigen is used in testing suspected sera; but, in tests on cases which present a previous history of typhoid inoculation, O antigen should also be used, as the serum titer with this antigen shows a greater rise during the course of the disease than from inoculation.

Preparation of H Antigens

H antigens should be employed for detecting H agglutinins against various *Salmonella* species, particularly in suspected typhoid and paratyphoid infections.

Smooth motile strains of the organism should be used. Make transfers to several tubes of nutrient broth and incubate at 37°C. for 18 to 24 hours. Examine gram-stained smears and exclude growths showing contamination.

Transfer 0.5 to 0.7-ml. quantities onto the agar slant surface in Blake bottles or Kolle flasks, being sure that the inoculum is well distributed over the surface. Proportionately smaller quantities may be prepared using agar slants. Incubate 24 to 48 hours at 37°C.

Examine as previously for purity and discard cultures showing contamination.

Wash off growths with a minimum quantity of sterile isotonic saline containing 0.5 per cent formalin, being careful to avoid particles of culture medium.

Transfer suspension to a sterile Erlenmeyer flask containing a single layer of glass beads and mix well to obtain an even emulsion.

Wash three times by centrifuging the emulsion at high speed for ten minutes, each time pipetting, discarding and replacing the supernate with 10 ml. of the formalized saline. After the third washing resuspend in the formalized saline.

Store suspension in the refrigerator for 24 to 48 hours, after which test for sterility by transferring one loopful to several tubes of infusion broth, incubating 48 hours at 37°C., and examining for sterility.

If no growth is evident, store in the refrigerator until needed. This constitutes the stock antigen and is diluted for use.

For use dilute with sterile isotonic saline to approximate the density of tube No. 3 of the McFarland nephelometer (see Nephelometry). Such suspensions keep several months in the refrigerator and should be tested occasionally for gross contamination. This antigen may be employed for routine macroscopic or rapid slide (spot) tests.

Preparation of O Antigens

O antigens are necessary for detecting the presence of agglutinins of *Salmonella typhosa*, Proteus X_2 and X_{19}. Two methods are available.

1. *Using a nonmotile strain.* A satisfactory non-motile strain of *S. typhosa* "0901" may be obtained either at the Standards Laboratories, Oxford or at the Lister Institute, England. A formalized saline suspension of a nonmotile strain is employed. The technic is the same as used for the preparation of H antigens.

2. *Using a motile strain.* (Slightly modified from Schaub and Foley after the method of Bien.) The preliminary technic for obtaining sufficient growth of a pure culture of a motile strain is similar to that used for the preparation of H antigen. After this has been obtained:

Wash off growth by adding a small amount of isotonic saline and adding an equal volume of absolute alcohol while agitating with a sterile glass rod which has been rounded on the end to prevent breaking off clumps of agar.

Incubate 12 to 24 hours at 37°C.

Mix well and leave at room temperature 2 to 3 hours.

Transfer to sterile container and add $\frac{1}{2}$ volume of sterile isotonic saline to reduce the alcoholic concentration 33 per cent, and store in refrigerator. This constitutes the stock solution and under refrigeration will keep indefinitely. Check culturally for sterility.

For use, dilute 1:6 with isotonic saline.

Other *salmonella* tests are performed on the blood serum of individuals or carried out in a manner similar to those outlined above. Antigens are prepared as described for H antigens for *S. typhosa*.

Paratyphoid fever is usually due to *S. paratyphi*, *S. schottmülleri*, or *S. hirschfeldii*, especially *S. schottmülleri*. Among the most frequent causes of other *Salmonella* infections producing gastroenteritis are *S. enteritidis*, *S. typhimurium*, *S. cholerasuis*, *S. newport* and *S. kentucky*.

Agglutination tests with patient's serum for paratyphoid fever are usually conducted with antigens of *S. paratyphi*, *S. schottmülleri* and *S. hirschfeldii*. Such tests may be performed microscopically with living cultures as above described. Normal sera may agglutinate in final dilutions up to 1:20 or 1:30. Agglutination at 1:40 is suspicious and at 1:80 or higher diagnostic in the case of individuals who have not been immunized with T.A.B. vaccine.

It is better, however, to conduct such tests according to the slow macroscopic technic, using H and O antigens prepared as previously

described. Normal sera may agglutinate the H antigens at about 1:20 and the O antigens in final dilutions of about 1:80.

Agglutination of an H antigen at 1:40 is suspicious and 1:80 or higher is definitely indicative of paratyphoid fever in individuals who have not been previously immunized. The same is true of agglutination of an O antigen at 1:160 or higher under the same conditions.

Cross-agglutination reactions with H and O antigens of the various Salmonella are commonly observed, but as a general rule, the titers will be highest for both the H and O antigens of the particular Salmonella-producing infection, especially the H antigen. Previous immunization with T.A.B. vaccine, anamnestic reactions and the carrier state, influence the reactions in the same manner as described in agglutination tests for typhoid fever.

Salmonella schottmülleri and S. paratyphi. The method of preparation of suspension is the same as that described for H antigen of *S. typhosa.*

Proteus X. The well known strains of Proteus reacting with the sera of various rickettsial diseases are: OX_{19}, OX_2, OXK. In this country, strain OX_{19} is of most importance as an aid in the diagnosis of typhus or spotted fevers. While sera from cases of typhus and spotted fevers agglutinate Proteus OX_{19}, differentiation may be made on a basis of specific complement fixation tests.

Since flagella (H) agglutinins are occasionally present in the sera of healthy individuals, it is necessary to use O antigens in performing the agglutination test. This antigen contains the fraction which reacts specifically with sera from typhus and spotted fever cases. Therefore, only smooth O strains should be employed.

Cultures are grown on *dry* slants of nutrient beef infusion agar pH 7.4. Since moisture increases the possibility of development of motile H forms, dehydrate the slants sufficiently to remove most of the water of condensation. An excess of moisture also tends to inhibit the growth of certain strains. Lyophilization of cultures is recommended.

Tests are best performed with fresh living cultures. In order that the foregoing specifications may be met, Gradwohl recommends the following procedure. The organisms are thinly inoculated on the dry surface of agar in Petri dishes and incubated 24 hours at 37°C. From these plates, smooth, non-spreading O type colonies are transferred to tubes of broth and slanted agar. If at the end of 24 hours incubation, the cultures are non motile, they are used in the preparation of antigen OX_{19}. Living organisms are suspended in physiologic saline until the

degree of turbidity is produced corresponding to tube No. 3 of the McFarland nephelometer. The antigen is now ready for performance of the macroscopic agglutination test, similar to that for typhoid fever.

In order to exclude spontaneously occurring variants, agglutination tests should be made at least once every three months with five specific antisera, *Salmonella typhosa*, *Salmonella schottmülleri*, *Salmonella paratyphi* and *Shigella paradysenteriae* Army and Flexner.

Shigella dysenteriae, *S. sonnei and S. paradysenteriae* (Flexner). In this test, a culture of the unknown organism is usually tested against the immune sera of three common types of dysenteriae—Shiga, Sonne and Flexner. It may be necessary to first differentiate between typhoid, paratyphoid and the polyvalent dysentery bacilli. Proper immune sera may be prepared or purchased from biological supply houses. Maintain stock cultures on agar (or bouillon). Suspensions should be prepared from S-strains as described for the H antigen of *Salmonella typhosa.*

Tests using the patient's serum are of little or no value in the diagnosis of acute bacillary dysentery, but may be of diagnostic aid in chronic cases of the disease.

Formalized antigens of Shiga, Flexner, Newcastle and Sonne strains of the Shigella and the slow macroscopic agglutination test are to be preferred.

Certain antigens of the *Shigella* are unagglutinable until heated. *Shigella alkalescens* for example, should be placed in a boiling water bath for 30 minutes before being used as an agglutinating antigen.

Positive reactions in a final dilution of serum 1:40 or higher with an antigen of *S. dysenteriae* are of diagnostic import. With antigens of *S. paradysenteriae* (Flexner), *S. sonnei* and the Newcastle strain, positive reactions in a final dilution of serum 1:160 or higher may be regarded as diagnostic.

Where agglutination tests are used for the purpose of diagnosing Shigella infections from feces, etc., polyvalent serum may be employed or monovalent sera for respective types. Technic is similar to that used for *S. typhosa.*

Flexner and Sonne types are most frequently encountered and cannot usually be identified readily. The degree to which further differentiation within the paradysenteriae group is carried out depends on circumstances. Such information may be unimportant clinically, but may furnish valuable epidemiological information.

Preparation of Brucella Antigen

An avirulent strain of *Brucella abortus*—such as strain 456, obtainable from the National Institute of Health, Bethesda, Md.—may be used for agglutination tests against all three species because of the fact that *Brucella abortus*, *Brucella suis* and *Brucella melintensis* are antigenically almost identical.

The antigenic suspension is prepared in a manner similar to that described for H antigen, with the exception that growth is obtained on trypticase soy agar using a 48-hour soy broth culture for inoculum. (Dehydrated trypticase soy agar and broth may be obtained from the Baltimore Biological Laboratories, Inc., 1640 Gorsuch Avenue, Baltimore 18, Md.) Either formalized or phenolized isotonic saline may be used for washing growth from the medium. The suspension is washed 3 times, as in the preparation of H antigens, and is then refrigerated. Sterility tests are made and the material is diluted for use to approximate that of tube № 3 of the McFarland nephelometer.

Preparation of Pasteurella tularensis *Antigen*

An avirulent strain (№ 38) of this organism has proved very satisfactory for routine tests. Its characteristics are that of a smooth, easily cultivated, highly agglutinable strain, and it is generally specific in its reactions. This strain may likewise be obtained from the National Institute of Health, Bethesda, Md. The organism is initially grown on cystine blood agar slants at 37°C. for 48 hours, after which 1 to 2 ml. of sterile isotonic saline are added to each tube and an even suspension obtained as in the preparation of H antigens. Blake bottles or Kolle flasks containing cystine blood agar are inoculated with 0.2 to 0.3 ml. of this suspension and incubated for 48 hours at 37°C. The growth is then washed off with formalized saline as in the preparation of H antigens and is washed 3 times with this by centrifuging, following which it is stored in the refrigerator, tested for sterility and diluted for use to the density of that described for the preparation of *Brucella* antigen.

Technic of the Macroscopic Tube Agglutination Test.

Material Needed. Small test tubes (12 x 75 mm.). Serologic pipettes (graduated 1/100th to tip). Rubber bulbs to fit pipettes. Tube rack. Water bath. Reagents.

Procedure:

Prepare the following set-up with all measurements in milliliters:

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7
Saline	0.9	0.5	0.5	0.5	0.5	0.5	0.5
Serum	0.1						
Mix well and transfer to succeeding tube	0.5	0.5	0.5	0.5	0.5	0.5*	
Serum dilution	1–10	1–20	1–40	1–80	1–160	1–320	Control
Add bacterial suspension	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Final or test dilution†	1–20	1–40	1–80	1–160	1–320	1–640	Control

*Discard 0.5.

†Higher dilutions may be made in a like manner as desired.

1. *Temperature.* The optimum is 50 to 55°C. in water bath; 37°C. or even room temperature are admissible for large-clumping, motile bacteria when 50 to 55°C. is impossible. For small-clumping suspension; 37°C. is not desirable.

2. *Time.* a. *For motile bacteria.* Two hours at 50 to 55°C., 8 hours at 37°C., or 24 hours at room temperature.

b. *For non-motile bacteria.* Four to twenty-four hours at 50 to 55°C., or 24 hours at 37°C.

c. *For O suspensions.* Twenty-four hours at 50 to 55°C. 3. *Reading*—Fig. 11.6.

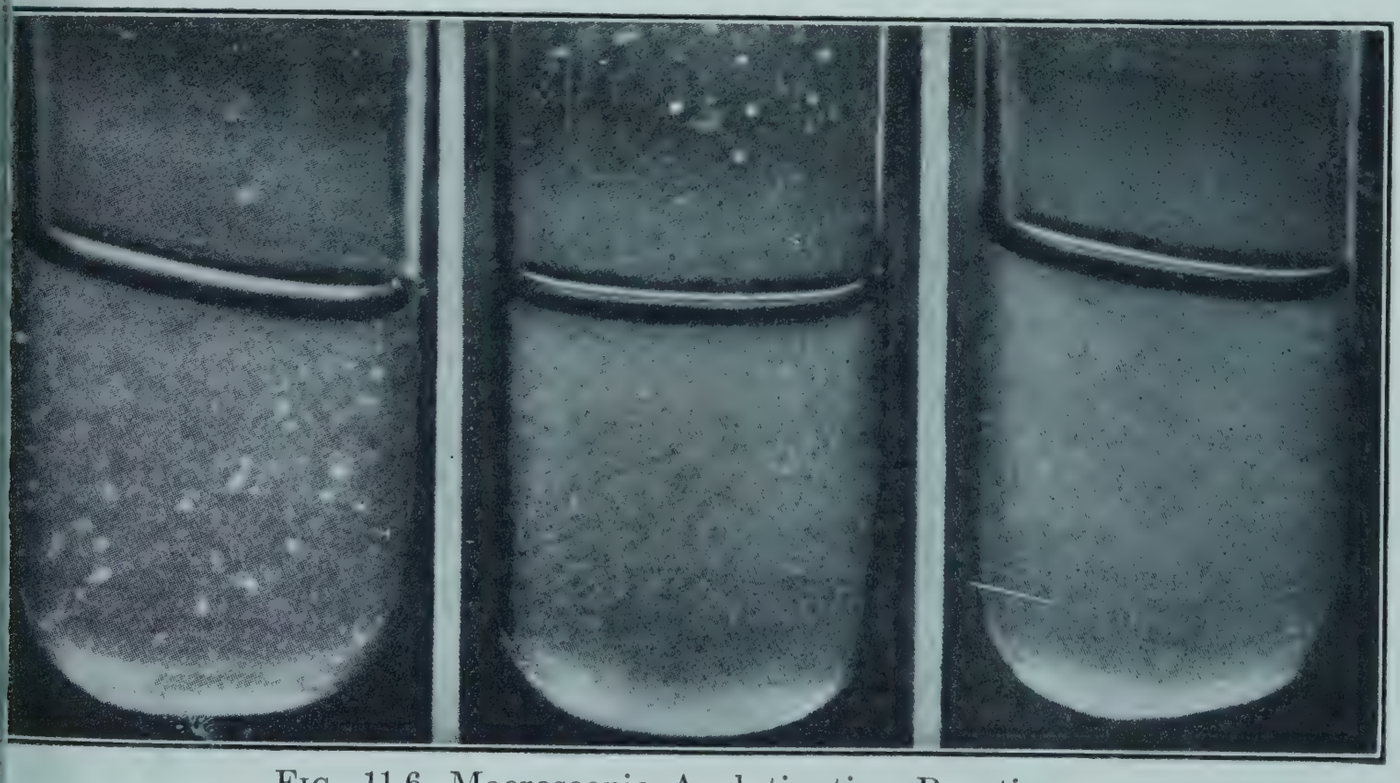


Fig. 11.6. Macroscopic Agglutination Reaction

1. Course flocculation (H-type). 2. Fine flocculation (O-type). 3. Control homogeneous suspension of bacteria in saline. (After Zinsser and Bayne-Jones: Textbook of Bacteriology, Appleton-Century Co.)

1. Preliminary reading of agglutination in 2 hours, final in 24 hours.
2. Control tubes should show no clumping.
3. Negative (−) tubes show no clumping.
4. Positive reactions may be read and recorded for each tube as follows:

   Complete agglutination with perfectly clear supernatant fluid = 4+

   Marked agglutination with slightly turbid supernatant fluid = 3+

   Moderate agglutination with turbid supernatant fluid = 2+

   Slight agglutination with turbid supernatant fluid = 1+
5. Notation made whether agglutination is floccular or granular type.

With phenolized, heat-killed and formalized antigens the agglutinated microorganisms occur as flocculent sediments; with alcoholic antigens the sediments are granular and less voluminous. To examine sediment, hold the tube rigid at the top and gently tap the bottom just sufficient to stir up the sediment which appears as masses or clumps in positive reactions. Too much agitation may re-suspend the antigen.

6. The highest final dilution of serum showing a 2+ reaction may be regarded as the titer.

The H type antigens, when agglutinated, show large, soft, snowy flakes. The O antigens give a more discrete, granular precipitate.

Prezones of non-agglutination may occur in the very low dilutions (1–20 or 1–40).

Turbidity controls prepared as indicated below may result in greater

Turbidity Controls for Reading Agglutination Reactions

Salt Solution	Agglutinating Suspensions	Reading
ml.	*ml.*	
0.5	0.5	−
0.625	0.375	1+
0.75	0.25	2+
0.875	0.125	3+
1.0	0.0	4+

uniformity of reading agglutination reactions. These are prepared and incubated with the test.

Nephelometry. Barium Sulphate Standards (McFarland):

To prepare, use 1 per cent sulphuric acid and a 1 per cent solution of barium chloride, both of A.C.S. purity. Solutions are made with distilled water.

Standard	1 per cent $BaCl_2$	1 per cent H_2SO_4
	ml.	*ml.*
No. 1	0.1	9.9
No. 2	0.2	9.8
No. 3	0.3	9.7
No. 4	0.4	9.6
No. 5	0.5	9.5
No. 6	0.6	9.4
No. 7	0.7	9.3
No. 8	0.8	9.2
No. 9	0.9	9.1
No. 10	1.0	9.0

Prepare fresh suspensions each year.

Select 10 tubes (150 x 19 mm.) of the same internal diameter and place in succession the amount of $BaCl_2$ and H_2SO_4 indicated and seal over a blow torch flame.

The calibrations of these standards in terms of the number of bacteria per ml. is accomplished by making direct counts of the organisms in suspension matching in density of several of the tubes in this series.

The standards having been established, it is an easy matter to determine the number of bacteria per ml. in any suspension.

The density of the tubes prepared as above corresponds approximately to bacterial suspension per ml. as follows:

No. 1: 300,000,000
No. 2: 600,000,000
No. 3: 900,000,000
No. 4: 1,200,000,000
No. 5: 1,500,000,000
No. 6: 1,800,000,000
No. 7: 2,100,000,000
No. 8: 2,400,000,000
No. 9: 2,700,000,000
No. 10: 3,000,000,000

Microscopic Slide Tests

While the microscopic, Widal or agglutination test has been employed for typhoid and paratyphoid fevers, it may be used, where indicated, for other types of infections. This test, however, has been largely re-

placed by the macroscopic slow agglutination test, using formalized (flagellar or H) and alcoholic (somatic or O) antigens which constitutes what is known as the refined Widal test. Living cultures are usually employed in the microscopic tests but heat killed or formalized cultures may be used. Cessation of motility where living cultures are employed is only an added advantage in reading the test, but the actual criterion is agglutination of the organisms. When living cultures are used care must be exercised that slides, cover glasses, etc. are placed in a suitable disinfectant solution or boiled for 5 minutes before handling and cleaning. Tops of working tables should be wiped with the disinfectant solution employed.

The use of serum is preferable although some laboratories are called upon to run microscopic Widal tests from specimens of dried blood which has been collected on glass slides or glazed paper. Where serum is used an initial dilution of 1:20 is prepared in a manner similar to that employed in the macroscopic tube agglutination test using measured quantities or drops.

Where dried blood is employed it is dissolved with a quantity of physiologic saline calculated to give the desired dilution. Dried blood in drops or smear may be assumed as containing a half volume of serum. Whether serum or dried blood is used the procedure for performing the tests is practically identical.

Freshly prepared 18- to 24-hour cultures of smooth strains of the organism employed are used for antigen.

A. Using Serum

Procedure:

1. Place 2 small watch glasses upon a sheet of white paper and designate on the paper, from left to right, A and B.

2. Using an ordinary medicine dropper or capillary pipettes add to A one drop of serum and 19 drops of saline. Follow by thorough mixing. After mixing add one drop of A to watch glass B followed by one drop of saline. This gives an approximate dilution as follows: A, 1:20, B, 1:40.

3. Place 1 loopful of bacterial emulsion in the middle of each of 3 coverglasses.

4. To the first mix 1 loopful of serum diluted 1:20, to the second 1 loopful of serum diluted 1:40 and to the third one 1 loopful of saline. This gives a final serum dilution of 1:40, 1:80 and a saline antigen control.

5. Mount each in vaseline on a hanging slide.

6. Set aside for 15 to 30 minutes at room temperature and examine under the high dry lens for clumping of the organisms. If there is no evidence of clumping incubate 1 hour at 37°C. If there is still no evidence of clumping the reaction is considered negative.

B. Using Dried Blood

While serum obtained from venepuncture allows for more accurate dilutions and is preferable when tests for several organisms are desired, blood may be obtained from the finger, or ear lobe. A few drops are gathered upon a clean glass slide or sheet of glazed paper and allowed to dry in the air.

Procedure:

1. A few drops of physiologic saline are added to the specimen and allowed to stand a few minutes to dissolve the clot and serum. With an inoculating loop mix specimen and saline at the outer edge of drop, avoiding as much blood as possible, until a loopful of the mixture held against a white background is a clear amber color. This gives an approximate dilution of 1:20. To a loopful of dilution 1:20 add one loopful of saline giving a dilution of 1:40. The procedure at this stage is similar to that outlined under *A. Using Serum*, paragraph 4.

Determination of Reaction:

First, examine the control containing only organisms and saline. This should show motility with no clumping. Then examine the serum-bacteria mixtures for clumping and record. For differentiating a negative, positive and pseudoreaction see Figure 11.7.

In the field of a control slide containing organisms in saline, the organisms are evenly distributed. They are a little thicker around the periphery where they are possibly attracted by their want of oxygen.

In a field showing a POSITIVE reaction, there are large clumps surrounded by a clear zone.

In a pseudoreaction (false agglutination), there is slight clumping. The clumps are small, somewhat evenly distributed and the organisms are relatively thick around the periphery. According to Park, pseudoclumping may occur with normal serum. This is more apt to occur in low dilutions when serum contains considerable hemoglobin and detritus. After one half hour, pseudoclumping tends to become quite marked. Fewer motile forms are present and more bacteria are present around the periphery and convexity of the drop.



FIG. 11.7. Microscopic Appearance of Hanging Drop Preparations in the Widal Agglutination Test for Typhoid.
a. Control slide. b. Positive reaction. c. Pseudoreaction (false agglutination). (After Wilson from Park and Williams: Pathogenic Microorganisms, Lea and Febiger).

Rapid Slide Agglutination Tests For Identification of Microorganisms

Enteric Pathogens

By means of this procedure tentative diagnosis may often be made directly from suspicious colonies developed on media used for isolating *Salmonella*, *Shigella*, etc. To carry out the test, place a drop of saline (control) on a slide and a drop of specific antiserum diluted (1:50 to

1:200) depending on the organism suspected. Sweep up the suspected colony with a platinum or nichrome loop, rub sufficient growth into the saline to give a grayish clouding then rub a loopful of this in the drop of the diluted serum. A positive result is shown by visible clumping in the serum drop. Spontaneous agglutination is controlled with the saline drop. Agglutination is rapid when positive. Check by observing under low power of microscope for clumping in the mixture of bacteria and antiserum and for smoothness of the emulsion in the salt solution control.

When a large number of cultures are to be examined a *screen test* may be made. This is done by emulsifying a portion of the suspected colony in two loopfuls of saline solution on a clean slide. A loopful of this is placed on the opposite end to serve as control. A loopful of immune serum is then added to the original mixture using a clean loop. The reaction is observed against a black background. The appearance of fine to coarse granules in the mixture indicates agglutination. No reaction should occur in the control. Negative reactions may be reported, but all positive ones should be retested by the macroscopic tube method.

Caution: It should be borne in mind that clumping of live organisms does not necessarily kill; therefore, coverslips or slides should be dropped in a jar of bichloride or boiled. The hands should be thoroughly washed, and the desk top washed with bichloride or other suitable disenfectant when tests are completed.

The rapid slide method may also be used for the detection of unknown sera, using a known antigen to determine the presence or absence of specific antibodies. An example of this is seen in the macroscopic plate agglutination test of Huddleston.

Rapid Slide Agglutination of Suspicious Meningococcus Colonies

Make separate suspensions of each of four suspicious colonies in 0.2 to 0.3 ml. of physiologic saline containing a drop of formalin, and mix well. Transfer two loopfuls of each to a clean glass slide. Near three of the antigen deposits place a loopful of Polyvalent types I–III and IIA antiserum respectively. Near the fourth loopful place one loopful of normal serum to serve as a control.

Flame and cool an inoculating loop; mix sera and antigen; flame and cool loop between each set.

Examine at once over strong light for agglutination. If this occurs the agglutination test is positive and the colony *Meningococci*. If no agglutination occurs the organism may be *N. catarrhalis* or *N. sicca*.

Macroscopic Slide or Plate Agglutination Tests

The Huddleson Rapid Method for Brucella Infection:

This test may be used for the detection of brucellosis in both man and animals. It is simple, rapid and reliable, and requires a single antigen made from an S. strain of *Br. abortus*. Huddleson states that there is no advantage in the use of separate or combined antigens made of each of the three species, as an antigen made of an S. strain of *Br. abortus* is satisfactory for detecting agglutinins produced by all three species of *Brucella*.

Antigen may be prepared by the method of Huddleson but may be purchased in 20-ml. vials, which includes a standard antigen pipette, from manufacturers of biological supplies.

Apparatus and Materials:

Serum pipettes. The standard 0.2-ml. pipette graduated in 0.01 ml. is preferable, as this size necessitates only one pipetting for each serum sample.

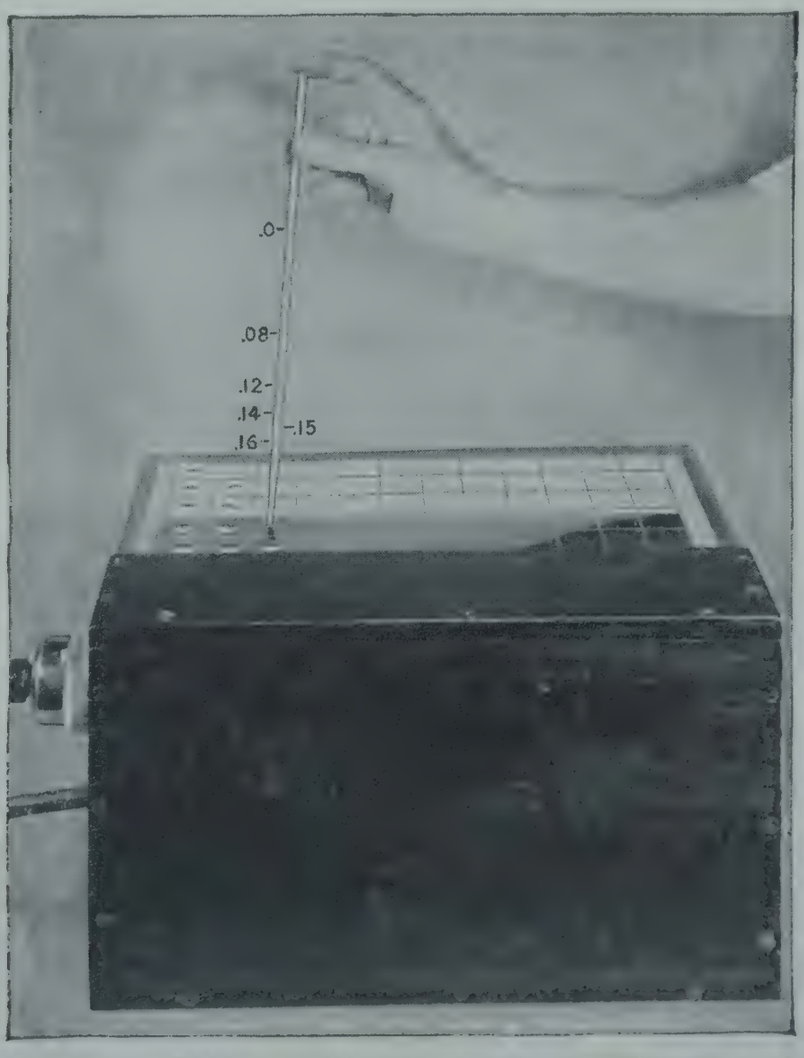


FIG. 11.8. Ruled glass plate and illuminator box. Showing placing of serum. (After Huddleson from Kolmer and Boerner: Approved Laboratory Technique. Appleton-Century Co.)

Standardized Dropper for Antigen. If one prepares antigen, it will be necessary to make the pipette. If purchased, a pipet is furnished. The pipette may be prepared by drawing out thick walled glass tubing of $\frac{1}{8}$ inch bore and cutting the capillary end at 0.07 or gauge 15 as measured by a U. S. standard gauge 283. The delivery end should not be flamed but should be left square. The outside diameter of the pipettes governs the size of the drop. Such pipettes delivers approximately 0.03 ml. of antigen per drop.

Glass Plate. This is made from window glass of double thickness ruled with Gold Seal or other laboratory ink into inch squares, five in a row (Fig. 11.8). Best results are obtained by testing no more than six sera at a time.

Dark-field Illuminator Box (optional) (Fig. 11.8). While this is convenient, readings may be made if the glass plate is placed upon a flat black background.

Toothpicks. For mixing serum and antigen.

Serum for Examination. Unheated, but brought to room temperature, about 25°C., before the test is begun. Antigen should be thoroughly shaken before use.

Procedure:

Using a graduated pipette, place each of the five amounts of serum on a square of one of the rows on the ruled plate, beginning at the top or bottom of the row, working across to the opposite side (Fig. 11.9).

Successive pipette readings required for each successive amount of serum are:

Square No.	Volume	Pipette Readings
	ml.	
1	0.08	0.08
2	0.04	0.12
3	0.02	0.14
4	0.01	0.15
5	0.004	about midway
		0.15–0.16

With the standard antigen pipette, place a drop of rapid antigen to each amount of serum. In delivering both serum and antigen, care must be taken to hold pipet and dropper in a vertical position, as holding at an angle will make considerable difference in the amount of serum and of antigen delivered.

When addition of the antigen to all sera has been completed, mix,

using a clean toothpick for each serum-antigen combination, starting at the smallest amount and working upward to the largest. On completion of this, lift plate from its position on the box, or flat surface if box is not used, and slowly tilt back and forth for two to three minutes. Then place plate in its former position, turn light on for illumination and read results.

Determination of Reaction:

Using ml. amounts of serum indicated and one drop of antigen (appx. 0.03 ml.), the final dilution shown below is attained. Dilutions are calculated on the basis of the old method when the amount in each tube was 2 ml.

Serum	Antigen	Final Dilution
ml.	*ml.*	
0.004	0.03	1:500
0.01	0.03	1:200
0.02	0.03	1:100
0.04	0.03	1:50
0.08	0.03	1:25

The reactions stand out very clearly as shown in Fig. 11.9. In the row of squares containing the designated amounts of serum, sample 1, is shown a negative reaction. No flocculation of the antigen has taken place. In the second row from the left, 0.08 ml. of serum of sample 2, has completely agglutinated the antigen and in 0.04 ml. the antigen has been partially agglutinated. The antigen shows complete agglutination with 0.08 and 0.04 ml. and partial with 0.02 ml. of serum sample 3. The antigen shows complete agglutination with three amounts of serum sample 4, with four amounts of serum sample 5, and with 5 amounts of serum sample 6. The agglutinated antigen in 0.004 ml. of a strongly positive serum, such as sample 6, often becomes very viscous and does not separate into individual clumps such as is seen with larger amounts of the same serum.

The light should be turned off as soon as the results have been read to prevent the plate from becoming too warm and thus cause too rapid drying.

Complete agglutination in 1:25 or over is positive.

Positive agglutinations occur in relatively few cases (about 5 per cent of those actively infected, and about 40 per cent of those who are immune). Positive reactions are more frequent in the active stage after convalescence begins. A negative reaction does not rule out the disease.

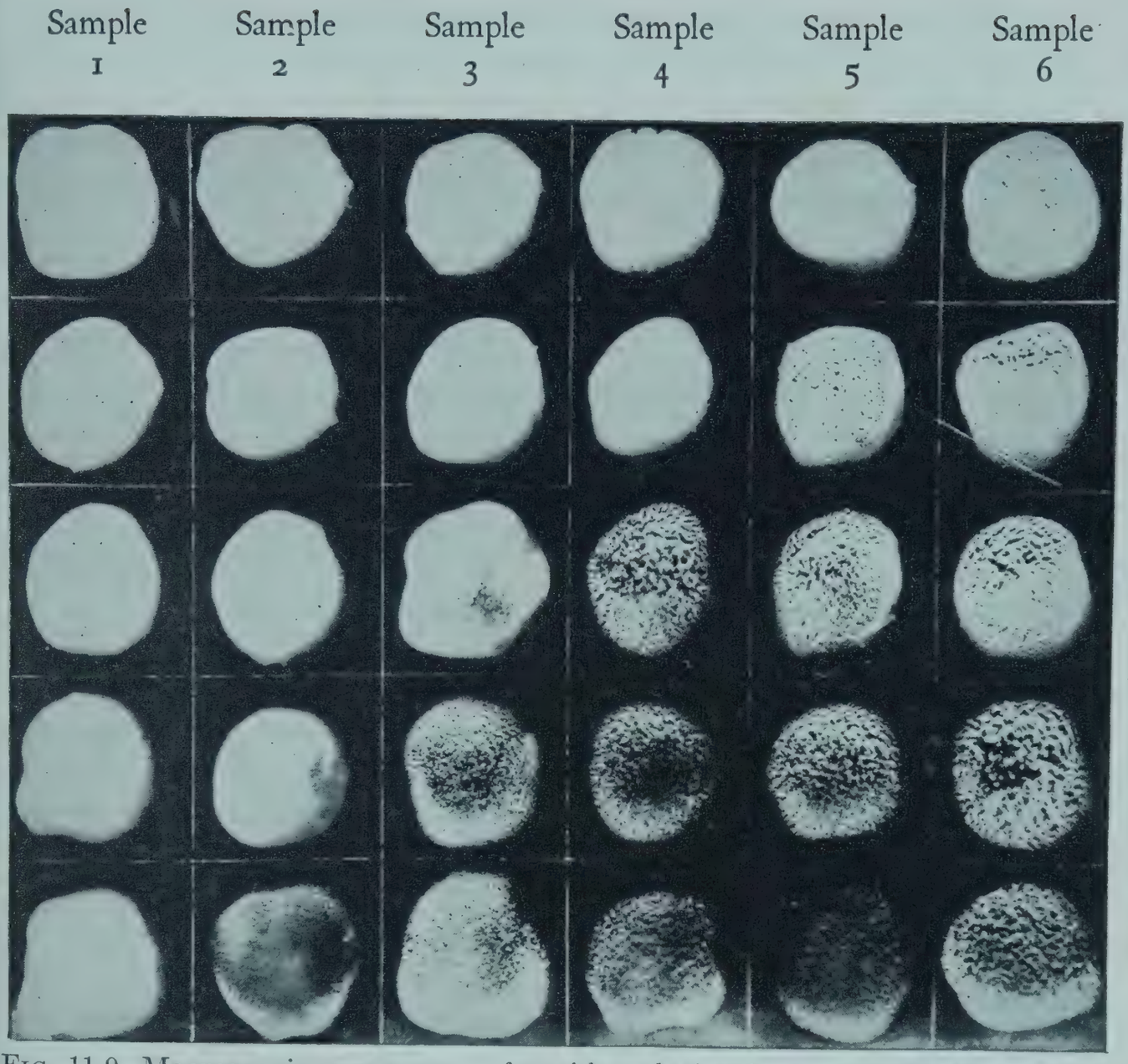


FIG. 11.9. Macroscopic appearance of rapid agglutination test. (Huddleson: Brucellosis in Man and Animals, The Commonwealth Fund.)

Detection of Vi Agglutinins for Typhoid Carriers

(Modified after Coleman in Diagnostic Procedures and Reagents, 3rd Ed., A.P.H.A., Inc., 1950; and Kolmer, Spaulding and Robinson: Approved Laboratory Technique, 5th Ed., Appleton Century Crofts, Inc., 1951.)

Vi antigens and agglutinating antibodies have previously been considered and described. The demonstration of the presence of Vi agglutinins in the blood serum of typhoid carriers is a most reliable method of detection and requires a special strain of *Salmonella typhosa*, rich in Vi and poor in O antigen. Bhatnagar's Vi 1 strain fulfills these conditions.*

* This may be obtained from the Communicable Disease Center, Enteric Bacteriology Laboratories, P.O. Box 185, Chamblee, Georgia.

Special precautions are necessary in order to maintain Vi cultures. Maintain a pure culture on inspissated egg media, of which the basic formula is:

Egg fluid (whole)	750 ml.
Water or other diluent	250 ml.

Mix thoroughly and dispense in sterilized tubes or screw cap vials and sterilize by inspissation in a slanted tube at 80°C. for one hour on two successive days. Since Vi antigen is very labile special care must be taken to prevent loss or variation during sub-culture. Inoculate several slants with a single stroke of the needle from base to top of slant. Incubate 15 to 18 hours at 35 to 37°C., seal, and store immediately in refrigerator. Fresh transplants should be made at 6-month intervals, the previous ones being discarded. Prepare antigen for agglutination test by making a sub-culture from one egg medium slant to beef infusion agar on several successive days. Incubate 18 to 20 hours at 35 to 37°C. Suspend growth from the agar slant in physiologic salt solution and adjust turbidity to correspond approximately to that of barium sulfate No. 3 standard. Prepare living suspensions each day that test is performed and keep in refrigerator if not used immediately, since the Vi antigen is altered fairly soon when kept at room temperature. A new antigen is needed each time agglutination tests are to be made.

Pure Vi strains should be stored in the dry state by lyophilization in order that they may be available if cultures on egg medium become unsatisfactory. The procedure for the test according to that outlined by Kolmer, Spaulding and Robinson is carried out as follows:

Prepare saline dilutions of the test serum in a series of 5 small test tubes (75 x 10 or 100 x 13 mm.), place 0.4 ml. of saline in tube 1 and 0.5 ml. in the remaining 4, as well as in a 6th (control) tube. Add 0.1 ml. of serum to tube No. 1, mix and transfer 0.25 ml. to tube No. 2. Repeat through tube No. 5 from which 0.25 ml. is discarded.

Add an equal volume (0.25 ml.) of antigen to all tubes. Control test by including a known Vi serum and a serum known to contain H and O antibodies but not Vi antibody. Incubate 2 hours at 35 to 37°C. and refrigerate overnight.

Examine for granular O type agglutination. If little or none is seen, centrifuge tubes for 10 minutes at 2,000 rpm. Shake gently and examine. Then compare reactions with those in the controls. A 2+ or stronger reaction in the first tube (1:10 dilution) is probably significant, since Vi antibody is rarely encountered in the absence of *Salmonella typhosa*. Repeat test in 5 to 10 days for rise in titer.

Knowledge of Vi antigen and antibody has proven of great value in the detection of chronic typhoid carriers among food handlers. Carriers not only have a higher agglutination titer in their blood than normal individuals but almost invariably have Vi antibodies in their serum. On the other hand Vi agglutinins are very rare in the blood of normal individuals.

Vi antibodies are increased very little by antityphoid vaccination.

In an uninoculated population a positive Vi agglutination is of definite diagnostic value.

In searching for typhoid carriers negative cultural findings are inconclusive but the presence of Vi agglutinins in the blood serum is significant.

In the laboratory diagnosis of typhoid fever it is unnecessary to use Vi antigen since Vi agglutinins are rarely present in the blood of such patients in the absence of H or O agglutinins. Likewise Vi agglutinins may not demonstrable in the blood serum of patients suffering from typhoid fever.

For complete consideration of Vi antigen and antibodies the following references may be consulted: Felix *et al.*: J. Hyg., p. 421, 1935; Pijper and Crocker: J. Hyg. Camb., 37: 332, 1937; Bhatnagar: Brit. M. J., 2: 1195, 1938; Ibid: J. Hyg., 38: 663, 1938; Horgan and Drysdale: Lancet, 1084–85, 1940; Eliott: Am. J. Hyg., Sect. B, 31: 8–15, 1940.

The Hemophilic Group

Hemophilus pertussis. Identification is made by means of agglutination and complement-fixation tests, organisms being recovered from cough plate cultures. Diagnosis by these methods is somewhat limited because antibodies usually do not appear in sufficient titer until the disease is in a moderately advanced stage. As pointed out by Kendrick, Lawson and Miller, serological tests in this connection are of greater importance in ruling out pertussis infection in the study of atypical coughs of long duration, and also in the investigation of individual immunity resulting from natural infection or artificial immunization.

Slide Agglutination Tests: Suspicious colonies are suspended in a drop of saline on one end of a clean glass slide. On the opposite end several loopfuls of this suspension are mixed with *Hemophilus pertussis* antiserum diluted 1:500 or $\frac{1}{10}$ the titer of the serum. Almost immediate agglutination occurs if the colonies are *H. pertussis.* The original suspension of organisms in saline is a control and should also be examined. Examination may be macroscopic and is confirmed by examining micro-

scopically. Results are significant only if the saline suspension remains smooth and fails to show clumping.

Since diagnostic information in pertussis is limited by the use of serological tests, macroscopic tube tests, complement-fixation and opsonic tests will not be considered here. For detailed information, see chapters referring to these procedures in Diagnostic Procedures and Reagents, 3rd Ed. 1950, A.P.H.A.

Rapid Pertussis Agglutination Test:

This test described by Powell and Jamieson in 1941 is used for determining the degree of immunity following infection or artificial immunization. This test can be conducted with a drop of blood from the patient's finger or ear or with a drop of serum if the blood is drawn and sent to the laboratory.

Procedure:

Place a small drop of the blue pertussis antigen, comprising approximately one-fortieth ml., on a piece of hard surface cardboard or stiff paper about the size of a microscope slide and not readily wetted through.

With a toothpick, a drop of antigen is quickly spread to form a circle about one-half inch in diameter. A similar drop of blood or serum is added to the drop of antigen, and mixture stirred a few minutes, and the cardboard or paper holding the drops then rocked or tilted back and forth for a minute. Coagulation of the blood is prevented by the sodium citrate in the antigen suspension.

Results of reactions using serum and blood are shown in Plate 11.I.

Appearance of the Reactions:

The following description of the reactions is given by the authors. A positive result is readily observed as granulation or clumping of the blue antigen. This is seen against a reddish background if blood is used, and against a white background if serum is used. Too long agitation of the test drop mixture giving a positive reaction may cause the clumped antigen to form a blue ring at the margin of the drop. A negative reaction comprises a diffuse blue color modified with red if blood is used.

Following agitation and observation of the antigen-blood mixture for about one minute, the drop-mixture on cards or paper is set aside and allowed to dry without disturbing. After partial drying has taken place, additional agitation may produce artifacts in the negative reactions. Plate 11.I shows the general appearance of rapid pertussis agglutination tests when conducted with the serum and blood of persons

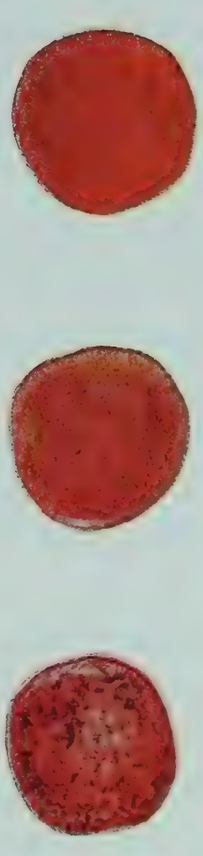


PLATE 11.I. Rapid Pertussis Agglutination Test (after Powell and Jamieson, Courtesy of The Lilly Research Laboratories). Upper: Negative. Middle: Weakly positive. Lower: Strongly positive.

showing negative, weakly positive, and strongly positive reactions. One by three inch pieces of glazed cardboard were used to hold the drop-mixtures shown.

The entire test may be conducted and read in two or three minutes, since only one minute's agitation suffices for the incubation period. The test card or paper holding the drop-mixture, after drying, comprises a permanent record which may be glued to the patient's record, or the original test may be done directly on a blank area of the record, if this is not quickly wetted through.

This test should be performed 3 or 4 months after primary vaccination has been completed. This lapse of time is necessary since circulating pertussis agglutinins develop slowly and do not reach maximum titers before 3 months. After this time they may perish considerably or completely disappear. No marked agglutination indicates a satisfactory response to vaccination. A negative agglutination test before 3 or after 4 months does not necessarily indicate lack of immunity. If the test is negative or only weakly positive, an additional dose of pertussis vaccine is indicated. To determine the results of secondary vaccination, the test should be performed 2 weeks after injection; the agglutinin responds to such stimulating of "booster" doses quite promptly, maximum levels being attained by the end of 2 weeks.

The test is of little value in the diagnosis of whooping cough since specific agglutinins develop late in this disease and seldom approach the high titers obtained by active immunization.

The antigen as supplied by Eli Lilly and Company, Indianapolis, Ind., consists of the 5-test package containing 5 capillary tubes of colored pertussis antigen and 5 comparator cards for reading the test.

Hemophilus influenzae. Six types of *Hemophilus influenzae* have been identified: a, b, c, d, e and f. Since almost all cases of influenzal meningitis are caused by type b *H. influenzae*, this type antiserum may be used for identifying and typing the organism directly from spinal fluid. The advantage in such identification is not only diagnosis of the causative agent, but typing permits the use of type-specific antiserum which is available for treatment.

Typing by Application of the Quellung (capsule swelling) Reaction:

The antiserum used in typing is prepared by hyperimmunizing rabbits with formalin-killed suspensions of selected strains of the organism.

Rapid diagnosis of meningitis caused by *H. influenzae* may be made by performing a Quellung test with the spinal fluid. If Gram-stain

smears show a pleomorphic Gram-negative bacillus, the test may be applied direct. If no organisms are found, the fluid should be centrifuged at about 2,500–3,000 R.P.M. for 20 minutes and the sediment examined. If organisms still cannot be found on smear, transfer 1 ml. of the fluid to Levinthal broth and 0.5 ml. to a Levinthal agar slant. Incubate both at 37°C. from four to six hours and examine by the capsular-swelling reaction.

Incubation of the spinal fluid itself (if the patient has not received sulfonamides) for several hours may produce sufficient growth of the organisms to make the test possible.

A large (4 to 5 mm.) loopful of *H. influenzae* typing serum is placed upon a clean coverslip. Care should be taken that the loop used must be cool before being placed into the serum vial.

Add 1 mm. loopful of dilute young culture or spinal fluid containing the suspended *H. influenzae* organisms to the loopful of antiserum on the slide. Then add a similar loopful of Loeffler's methylene blue dye and thoroughly mix.

Invert the coverslip on a clean plain glass slide, using sufficient pressure to make a thin even layer between the two glass surfaces.

Allow to stand five minutes and examine under oil immersion lens for dark blue rods or coccobacillary forms, surrounded by an unstained



FIG. 11.10. Quellung reaction of *H. influenzae* with diagnostic typing serum. (After Alexander: American Journal of Diseases of Children.)

area with definite outline. (Swelling of the capsule is often slow to appear, and it may be necessary to let the preparation stand for 30 to 60 minutes in a moist chamber at room temperature and then to re-examine.) Such will be seen only when the organisms present are of the same type as the serum used in the test.

In Alexander's experience, the Quellung reaction applied to the patient's serum may serve as a useful guide to the sufficiency of a given dose of antibody.

Agglutinin Absorption Test

(*Method of Zinsser and Bayne-Jones*)

The procedure is described in connection with the so called colon-typhoid paratyphoid group, but may be modified to apply to other groups of bacteria.

Procedure:

Absorption of agglutinin is used: (1) to determine relationships among members of a group of bacteria, and (2) to prepare strictly specific monovalent agglutinating sera.

The minimal absorbing dose of the homologous bacterium should be determined in each case and two or three times this amount should be used in absorbing the agglutinin from the serum. Final titrations with absorbed sera should be made over the whole range of the titer. Prezone reactions may inhibit agglutination in tests with 1–10, 1–40 and even 1–100 dilutions of the absorbed serum. Time of contact, shaking of mixtures, temperature of incubation, dilution of serum and a number of other factors influence the results of agglutinin absorption. The technic must be varied to suit each problem. A satisfactory procedure for absorption of agglutinins in antisera by members of the colon-typhoid-paratyphoid group is as follows:

1. Calculate the amount of serum required for the final titration.
2. Dilute sufficient of the antiserum 1:10 with salt solution.
3. Wash off the growth of an 18 hour culture of *S. typhosa* (or other member of the group) on a plain agar slant. To do this, add 2–3 ml. of salt solution to the tube, emulsify the bacteria in this by scraping them off the agar with a platinum loop, taking care not to break the agar surface. Place the emulsion of bacteria in a graduated tube and centrifuge this at 2000 R.P.M. until the volume of packed bacteria is constant. Pipet the supernatant saline.
4. To 1 part of the bacterial mass in the centrifuge tube, add 9 parts of serum diluted 1:10. This gives an approximate 1:10 absorbing dose

of bacteria. For exact method of computing this, see Krumwiede's paper.

5. Shake the mixture of bacteria and serum and incubate in water bath for two hours at 37°C. Refrigerate over night.

6. After incubation and refrigeration, centrifuge to remove bacteria and pipette the clear supernatant fluid.

7. Set up agglutination tests with the absorbed serum and broth culture antigens of the bacteria.

Example: Required: 2 ml. of absorbed serum diluted 1:10. Absorbing dose equals 0.2 ml. of packed bacteria. Then: to 0.2 ml. of packed bacteria in a graduated centrifuge tube add 1.8 ml. of serum diluted 1:10.

This gives approximate results. The formulae used by Krumwiede for this calculation, in which a correction factor is used for the saline included in the bacterial mass, are as follows:

$$(\text{Mass} - 10\%) \times \text{dose factor} = \text{total volume}$$

$$\frac{(\text{Mass} - 10\%) \times (\text{dose factor} - 1)}{\text{serum dilution factor}} = \text{amount undiluted serum required}$$

Agglutination Test for Leptospira

This test is somewhat involved and requires living cultures of Leptospira, which are not easily cultivated or kept in living state. For details of this test, see Lab. Guide Bact., Naval Med. School, Nat. Naval Med. Center, 1944, and Diagnostic Procedures and Reagents, 3rd Ed., 1950, Am. Pub. Health Association, Inc.

Agglutination Tests for the Detection of Primary Atypical Pneumonia

See Chapter 10, Diagnosis of Viral and Rickettsial Diseases.

Isoagglutinins and Autoagglutinins

See Chapter 6, Blood Groups.

Heterophile Antibody Test

See Chapter 5, Diseases Primarily Involving Leukocytes.

Serological Test for Identification of Sporotrichosis Antibodies in Patient's Sera (Naval Med. School)

Procedure:

1. Obtain serum from coagulated blood of patient.

2. Make suspension of sporotrichum by grinding growth from week-old cultures with saline in a sterile mortar, and filter through cotton.

3. Adjust turbidity of suspension to approximate that of No. 2 barium sulphate standard (McFarland), using saline as diluent.

4. Prepare a series of serum dilutions starting with 1:20 and extending through 1:5,120, using 0.5-ml. amounts in agglutination tubes. Use 0.85 per cent saline as diluent

5. Add 0.5 ml. of the organism suspension to each serum dilution and also to a tube containing 0.5 ml. of saline, as control.

6. Shake rack vigorously for 1 minute.

7. Incubate at 37°C. for 2 hours and at room temperature overnight.

8. Read in comparison with the saline control.

Interpretation of Agglutination Tests

Typhoid and Paratyphoid Fever. Agglutination tests for typhoid and paratyphoid fevers are commonly called "Widal" tests. The exact manner of conducting the test is a matter of choice. The tube method is preferable in all cases of doubt and as a routine measure wherever facilities permit its use.

The positive Widal test should always be interpreted in the light of clinical findings. To do otherwise will only confuse the picture. Several things contribute to this situation:

(1) The common use of typhoid and paratyphoid vaccine has endowed many persons with specific agglutinins, which may subsequently be evidenced by a positive Widal reaction for from 1–5 years. A definite history of vaccination and employment of O and H antigens will prove useful in diagnosis. For early diagnosis, blood and urine cultures are of greater value than the Widal since bacteria may be found in 75 per cent of the cases during the first week.

(2) In regions where typhoid fever is endemic, "normal" agglutinins may be encountered in an appreciable number of the population.

(3) "Anamnestic" phenomena occur. By this is meant the formation of the agglutinins in persons suffering from fevers other than typhoid. The antibody forming capacity for typhoid may be reawakened as a result of the associated fever. Of course, the capacity to form typhoid antibodies must at some time have been established in the patient. In the anamnestic response the final titer usually is of a lower magnitude than that encountered in infection.

In a small percentage of persons no appreciable immunological response can be demonstrated. Some patients produce chiefly O-agglutinins. These may be overlooked unless an O-antigen is used in the test.

Individuals vary in their response to infection. Variation in titers is the rule rather than the exception. From this it should be clear that the application and interpretation of the Widal test to enteric infection requires a fine sense of discrimination and judgment. Every effort should be made to ascertain whether the patient has been inoculated against, or has ever had, the disease and if so to fix the time of such occurrence. It is necessary to know the duration of the present illness. With these facts in hand, an intelligent application of the agglutination test is possible.

In typhoid fever, the agglutinins begin to appear in the blood in from 7 to 10 days from the onset of illness and reach their maximum concentration in from 3 to 5 weeks. Attention should be paid to the rising titer, hence the Widal test should be applied as early as possible. In the absence of previous inoculation or attack of the disease, a rising titer up to 1:100 or more is strong indication of infection. When the patient gives a history of previous inoculation against typhoid within the past three years, the diagnostic titer should be advanced to 1:500 or over.

In the case of paratyphoid fever, an agglutination test with a smooth, motile strain of *S. paratyphi* may be considered positive in dilutions of 1:40 and up, in the absence of previous inoculation. The disease is less common, hence "normal" agglutinins are less frequently encountered. The antigenic response also seems to be less marked in this disease. In diseases caused by *S. schottmülleri*, positive Widal tests comparable to those in typhoid may be obtained. However, owing to the diphasic nature of this organism, as well as the similarity of the antigens of different species of the Salmonella group, the accuracy of the serological diagnosis suffers as a consequence. Negative tests may be obtained because of discrepancy in the phases of the infecting and the test organism. For this reason, it is best not to neglect cultural studies of the patient.

Knowledge that O antibodies are found early in disease, lost rather soon and are less highly specific while H antibodies are formed late, retained for long periods (years) and are highly specific, makes interpretation of Widal findings more precise where the "refined" test is employed.

The following points, set forth by Kolmer, are of material significance in evaluating results of such tests.

1. Normal sera may agglutinate H antigen at about 1:20 and O antigen in final dilutions up to about 1:80.

2. Agglutination of H antigen at 1:40 is suspicious and at 1:80 or

higher definitely indicative of typhoid fever in individuals who have not been previously immunized with typhoid-paratyphoid vaccine.

3. Agglutination of O antigen at 1:160 or higher is also indicative of typhoid fever in individuals who have not been immunized with typhoid-paratyphoid vaccine.

4. Active immunization with typhoid-paratyphoid vaccine produces both H and O agglutinins. The presence of O agglutinin in higher titer, with H agglutinin in low titer, is indicative of typhoid fever in a previously vaccinated individual. However, a very high titer of H agglutinin as, for example, 1:1280 or 1:2560, may be indicative of typhoid fever in a previously vaccinated individual since it is rather unusual for H agglutinin to persist in titers of more than 1:640 for longer than 6 months after immunization. Consequently, when the disease is suspected in a previously vaccinated individual the tests should be repeated every 3 to 5 days with the same antigens. If the titers progressively increase and especially for O antigen, typhoid fever is most likely present.

5. Acute infections like influenza, brucellosis, etc., in individuals previously immunized with typhoid-paratyphoid vaccine may produce a high titer of H agglutinin with a low titer of O agglutinin (anamnestic reaction).

6. In vaccinated individuals a drop in H, and especially in O, agglutinins to normal levels may be indicative of the advisability of reimmunization with typhoid-paratyphoid vaccine.

7. An increase of H, and especially of O, agglutinins is also of aid in the detection of typhoid carriers among nonvaccinated individuals. To be significant, the titer of H agglutinin must be above 1:20 and O agglutination above 1:100.

8. Because of the close relationship of the typhoid bacillus to the paratyphoid bacilli A and B, and especially B (*Salmonella schottmülleri*), agglutinins for the latter may also be increased in typhoid fever. However, the titer is usually considerable lower for the paratyphoid bacilli than for the typhoid bacillus. In case of doubt, the absorption test should be conducted.

Agglutination tests with patients' sera in gastro-enteritis due to *Salmonella* are usually of little or no diagnostic value. Such tests, however, as well as those in connection with other enteric organisms, are of extreme value in the final identification of the organisms recovered in cultures from stools and other sources. Such tests, particularly for the *Salmonella*, are best conducted with rabbit immune sera from which the

group agglutinins have been removed by absorption. Living cultures may be employed as antigens in microscopic tests; otherwise, H antigens are preferred for the conduct of macroscopic slow agglutination tests.

Bacillary Dysentery. Agglutination tests using blood serum of patients with known Shigella antigens are unsatisfactory since perceptible antibodies are not present until late in convalescence. The ultimate titer is usually not high and group agglutination is a common feature when present. Therefore examination of feces in the search for the organisms and confirmation by the use of Shigella agglutinating serum is the method of choice.

If one chooses to perform agglutination tests on the blood taken late in the disease the following points should be noted:

(1) A rising titer against *Shigella dysenteriae* in a dilution of 1:40 or over may be considered positive evidence of infection.

(2) Agglutination of a paradysentery strain in the absence of agglutinins for *Shigella dysenteriae* is suggestive if the titer exceeds 1:150.

(3) Normal agglutinins for *Shigella dysenteriae* are not common, but many persons may be expected to show agglutinins for one or more of the paradysentery group (Flexner, Hiss, etc., strains).

(4) Upon the termination of the disease, they tend to disappear from the blood within 3 to 6 months.

Typhus Fever. The agglutination test using *Proteus Vulgaris* OX_{19} is known as the Weil-Felix reaction and has been extensively employed in the diagnosis of typhus fever. A positive reaction is obtainable in epidemic and murine typhus, spotted fevers and other rickettsial diseases but is of limited differential value. The test is peculiarly non-specific in that there is no evidence at present to show that *Proteus vulgaris* OX_{19} is an etiologic factor in rickettsial diseases.

Formalized antigens of the OX_{19} (National Institute of Health No. 504), OX_2 and OXK strains of *Proteus* are commonly employed in the macroscopic slow agglutination test. Some laboratories prefer alcoholic antigens of these strains. Proper controls, consisting of a known positive human or rabbit immune serum and a known normal human serum, should always be tested at the same time. Normal human sera do not ordinarily agglutinate antigens of these strains in final dilutions higher than 1:25 to 1:50.

Positive agglutination reactions are obtainable in 95 per cent of cases in this country. The agglutinins are generally not demonstrable during the first week of illness, but may be found during the second week. While a single positive test may be misleading in cases of past infection,

a rising titer of 1:80 is significant especially when preceded by a lower titer. Prezone reactions may be encountered and dilutions should be sufficiently high to escape this fact. The test should be performed according to the technic of the macroscopic tube method with incubation at 37°C.

In most typhus fever cases the agglutinin titer is usually 1:25 on the fourth day and 1:50 or higher by the eighth day. The titer usually rises by the end of the second week to several thousands, even as high as 1:50,000 and declines rapidly during convalescence. The reaction may become negative within 5 or more months following recovery or may persist for several years.

In the spotted fevers the titer of 1:160 or higher usually occurs. Baker recommends that the test be conducted as soon as spotted fevers are suspected or when the corruption first appears with a second test following on or about the twelfth day and third during convalescence. A single positive test may be misleading in cases of past infection and the significant factor lies in a rising titer, expecially when preceded by a low initial titer.

Cross agglutination reactions in rickettsial diseases may occur with antigens of *Br. abortus*. Prezone reactions may be encountered in these tests, hence, dilutions should be sufficiently high to escape this fact.

Tests should be performed according to the technique of the macroscopic tube method for incubation at 37°C.

Agglutination reactions of *Proteus* in typhus, spotted fever and tsutsugamushi disease, taken from Felix, are indicated below.

Proteus	Typhus Fever	Spotted Fever	Tsutsugamushi
OX_{19}	+++	+	−
OX_2	+	+	−
OXK	−	+	+++

Tularemia. Agglutinins are generally absent from the blood during the first week of the disease, appearing in increasing concentration from the second week on to reach a maximum concentration in from 4 to 7 weeks. Titers of 1:1000 or over may be encountered. At the end of a year the titer is usually about 1:100. There is some evidence that tularemic agglutinins persist in the blood for long periods of time. Francis and Evans demonstrated a titer of 1:20 in a person eighteen years after infection.

Agglutinins for the *Brucella* organisms may develop in tularemia.

If the infection is primarily caused by *Pasteurella tularense* a higher titer for *Pasteurella tularense* should be present. The necessity for conducting parallel agglutination tests with both organisms in the absence of any clinical knowledge of the patient in question is quite apparent. The interpretation of tests for tularemia should be closely correlated with clinical data if possible.

In a study by Francis and Evans, an examination of 500 Wassermann sera yielded 20 positive reactions for *P. tularense* none of which exceeded 1:40. The same investigators noted an average titer of 1:155 in the second week of the disease; in the third week of 1:450. Cross agglutination with the *Brucella* was encountered in 37 sera out of 100 clinical cases of tularemia. Cross agglutination with the Brucella occurred only in sera which agglutinated *Pasteurella tularense* in dilution of 1:320 or over. For diagnosis, a titer of at least 1:80 should be present. A marked rise in titer is of great significance.

Undulant Fever. Two methods of performing the agglutination test to detect *Brucella* infection in human beings and animals are available: the test tube method and the rapid method. According to Huddleson, both have been subjected to critical study by many workers and are found satisfactory. Patients with undulant fever sometimes agglutinate *Pasteurella tularense* in addition to Brucella.

Investigations have shown that 0.5 per cent to 10 per cent of persons in certain groups harbor agglutinins for *Brucella abortus* in dilutions exceeding 1:100. This tendency increases with the liability to infection, particularly in those whose occupations bring them into intimate contact with farm animals. This has been definitely noted in veterinarians, slaughter house employees and inmates of institutions using infected milk. These agglutinins may be non-specific or they may indicate past or present infection. The titer factor, therefore, is of great importance.

In acute brucella infections agglutination tests show little or no titer during the first 10 days of the disease. As the disease progresses successive specimens ordinarily show a rise in titer reaching a peak sometime during the 3rd to 6th week and may subsequently show a decline. In some cases, the immunological response is very slight and may not be demonstrable for months, hence it is imperative that every means be used to establish a diagnosis. The agglutination tests form only one link in the chain of evidence.

A titer of 1:20 commencing in from 2 to 3 weeks after onset of illness and increasing to 1:100 or above may be considered positive evidence of this infection in the presence of clinical symptoms. There is no

"diagnostic" agglutination titer in brucellosis. In general, a titer of 1:320 or higher, in the presence of symptoms, is presumptive evidence of illness. Higher titers are frequently noted.

In chronic infections the significance of agglutination test titer cannot be judged due to lack of any definite criterion. However, no question is likely to be raised in patients yielding titers of 1:160 and higher combined with clinical findings and without symptoms of tularemia. Brucellosis cannot be ruled out on a basis of laboratory tests alone as well as any degree of agglutination is obtained in dilution of 1:20. Some individuals although infected do not develope detectable agglutinins or have lost them. Therefore negative reactions alone do not necessarily exclude brucellosis. Some sera may give negative tests because of the presence of a heat-labile blocking antibody which reacts as a univalent nonagglutinating antibody. Prozone reactions may also occur. Repeated blood cultures are helpful in such cases.

When a patient's serum agglutinates both *Brucella abortus* and *Pasteurella tularense*, the higher titer of the two points to the nature of the infection. Where both titers approximate each other or if a serum agglutinates *P. tularense*, *Br. abortus* and *Br. melitensis*, to the same, or nearly the same titer, agglutinin-absorption tests may be of service in differentiation. For technic, see Absorption Tests.

Pasteurella pestis. As applied to patient's serum, agglutination tests are of little value for early diagnosis as agglutinins do not appear in patients suffering from plague before the ninth day of the disease. Agglutination in dilutions of serum 1:20 to 1:40 or higher is of confirmatory value in convalescence or following recovery. Normal sera may agglutinate *P. pestis* in final dilutions up to 1:10.

If the test is employed phenolized or formalized antigens of *P. pestis* are used. Due to a strong tendency to spontaneous agglutination the macroscopic tube test is to be preferred to the microscopic.

Hemophilic Infections. These have been interpreted under the technic for each, as have the succeeding agglutination tests.

PRECIPITIN TESTS

These tests have as their basis a type of antigen-antibody reaction in which the antigen, a protein, is precipitated by a specific antibody, the precipitin. The fine character of the precipitate is due to the colloidal nature (small size) of the antigen particles. The actual mechanism of the flocculation is similar to that described for agglutination.

Specific precipitins are formed in the body and are present in the

blood serum as a result of infection by bacteria and animal parasites or may be produced at will in animals by injection of bacteria and certain types of foreign protein. This occurrence is utilized in tests for the diagnosis of trichinosis and echinococcus disease; for the species identification of blood (antihuman vs. human serum); and for the identification of types of various bacteria.

Beta Hemolytic Streptococci

When beta hemolytic streptococci are found in pure culture in specimens of purulent discharges, blood or spinal fluid, the etiologic relationship is, in general, established.

Determination of the serological group of beta hemolytic streptococci by the Lancefield method may not only yield valuable information to the clinician in epidemics and in treatment but, as pointed out by Goerner, Massell and Jones, may aid in the elimination of the carrier state, due to Group A hemolytic streptococci through use of adequate penicillin therapy.

Lancefield has grouped the beta hemolytic streptococci on the serological basis of the precipitin reaction. Twelve groups in all have now been differentiated (A–H, J–M). Group A constitutes most of the strains of hemolytic streptococci that are pathogenic for man. This group includes streptococci of septic sore throat and scarlet fever. Any of the groups, however, may be harbored by man with or without evident symptoms.

The five groups originally described are:

Group A—pathogenic for man

Group B—derived for the most part from cases of mastitis in cattle and is important epidemiologically

Group C—from various pathologic processes of the lower animals

Group D—isolated from cheese

Group E—isolated from milk

Milk frequently contains organisms of Group B, C, D and E. Those of Group D are resistant to pasteurization, hence the desirability of being able to make a rapid diagnosis of the streptococci found in human material or in milk is evident.

Lancefield and others have subdivided the primary groups into a number of types, determined in Group A by protein components (M-antigens) of the cell; in Group C a protein component, and in other groups a carbohydrate. The M and C substances are demonstrable by precipitin reactions.

The "M"-substance of Group A is lost during prolonged cultivation on artificial media and is regained only after repeated mouse passage.

Precipitin tests for grouping and typing of beta streptococci may be performed with the same antigen and if typing a strain is to be done it should be performed as soon as possible after isolation because of the probable loss of a type-specific M. "substance." However, cultures that are dried and frozen immediately after isolation may be typed at any time.

*Tests for Grouping and Typing Beta Hemolytic Streptococci**

As above stated, the same antigen may be used in both tests. Anti-sera for these tests may be obtained from the Lederle Laboratories, 30 Rockefeller Plaza, New York 20, New York.

These tests are performed with use of capillary tubing which has the advantage of being more easily read and economical in the use of sera. Two sizes are required. That for the grouping test with an outside diameter of 1.2 to 1.5 mm., that for the typing test with an outside diameter of 0.7 to 1.0 mm. These should be obtained in lengths of 7.5 cm. Greater lengths may be cut accordingly. The capillary tubing may be obtained from the Kimble Glass Company, Vineland, New Jersey.

When anti-serum and antigen are drawn into tubes they are conveniently arranged in a capillary pipette stand made of a wooden block approximately 12 inches long with a center groove filled with *Plasticene*.

Preparation of Antigen for Grouping and Typing

Following isolation the organisms should be grown for 18 to 24 hours in 10 ml. of Difco tryptose phosphate broth at pH 7.8 to 8.0. The culture is centrifuged, the supernant fluid discarded and the sediment resuspended in 5 to 7 drops of N/5 HCl. (Add 1 ml. concentrated HCl to 59 ml. physiologic saline.) This is well mixed with a wooden applicator stick and 1 drop of 0.01 per cent thymol blue indicator added. (0.01 gm. thymol blue in 60 ml. alcohol and 40 ml. water.) A peach color results.

Heat in a boiling water bath 10 minutes with occasional shaking, and cool in the refrigerator for 10 minutes. Follow by centrifuging.

Pipette the clear supernate into a clean test tube and add 1 drop of

* Modified from Schaub, Isabelle G., and Foley, M. Kathleen: Diagnostic Bacteriology, Fourth Ed., 1952, The C. V. Mosby Co., St. Louis; and Swift, Homer. F., Wilson, Armine T., and Lancefield, Revecca C.: J. Exper. Med. 78: 1943.

phenol red 0.01 per cent indicator. (0.01 gm. phenol red in 60 ml. alcohol and 40 ml. water.) The solution becomes a distinct yellow. Add buffer solution drop by drop until a pink color develops—pH 7.0 to 7.8. Buffer solution: Dissolve 1 gm. anhydrous sodium acid phosphate in 100 ml. of N/5 NaOH (0.8 gm. NaOH in 100 ml. distilled water). Centrifuge and pipette supernate, which must be crystal clear, into a clean sterile container. This constitutes the antigen.

Procedure for Grouping Beta Streptococci

The outside of a piece of 1.2- to 1.5-mm. capillary tube is cleaned with a piece of lens paper, dipped into Group A streptococcus anti-serum which is drawn up $\frac{1}{3}$ the length of the tube. The outside of the tube is wiped with tissue or lens paper to remove excess serum and prevent adultration of the antigen. The capillary tube is next dipped into the antigen and an equal amount of the extract drawn into the tube. The outside of the tube is wiped off and inverted until there is an air space both above and below the column of liquid. There should be an interface formed by contact of the anti-serum and antigen. If an air space exists between these a fresh tube should be prepared. The tube is then placed upright in the *Plasticene* contained in the wooden block. A properly prepared tube should now contain an air space, a column of serum layered over a column of antigen with no air bubbles interfering and a column of air above the *Plasticene*.

Similar tests are repeated using Groups B, C and D anti-sera. The

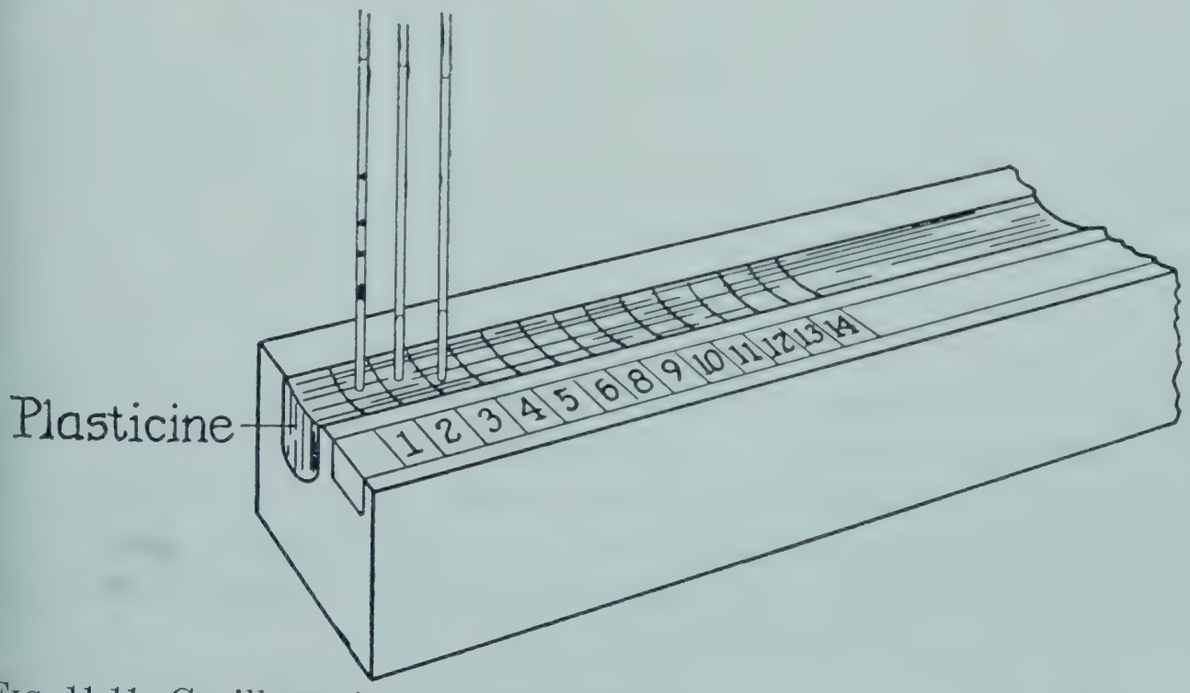


Fig. 11.11. Capillary pipette stand with three pipettes in place; precipitate in rst tube. From Homer F. Swift: J. Exper. Med. 78: 129, 1943.

capillary tubes should be examined with a hand lens immediately after the tests have been set up using a strong light in front of a black background. If the test has been correctly performed all tubes should be clear.

The tubes are left at room temperature and re-examined after 15 to 30 minutes. A milky ring will be formed in the tube containing antigen and homologous antiserum at the junction of these fluids. A positive reaction is sharp and definite. The ring disappears after an hour or two; then a white precipitate settles to the bottom of the liquid. In negative tests the liquids remain perfectly clear. No precipitate is evident.

If the organism is found to be that of Group A, and typing of the strain is desired, the same antigen as indicated above may be used. This extract may be kept in the refrigerator for at least a week with satisfactory results.

Procedure for Typing Beta Streptococci

The outside of a piece of 0.7- to 1.0-ml. capillary tube is cleaned with a piece of lens paper.

The same technic as described above is used, substituting beta streptococcus typing serum in place of the group serum. The tubes are examined immediately to detect any extraneous material that might interfere with the accurate reading of the test. The tests are incubated for 2 hours, refrigerated overnight and read as above described. In some instances it is possible to read the tests following incubation, in others it is necessary to refrigerate before a reaction is obtained.

Cross reactions may occur and in such cases may be eliminated by repeating the tests, using the sera in which precipitation has occurred and diluting such sera 1:2, 1:4 or 1:8 with isotonic saline. This is incubated for 2 hours, refrigerated overnight and read.

Anthrax

The precipitin reaction developed by Ascoli has had wide application in some European countries for detecting anthrax-infected dried hides. It may likewise be used for detecting anthrax specific antigens in materials of any kind. It is simple and rapid, and its only disadvantage lies in the difficulty in securing suitable antiserum. According to Hagan, most anthrax serum is not suitable for this test.

Procedure (Hagan):

The suspected materials are finely chopped and placed in a small amount of saline solution in tubes.

Tubes are then placed in the boiling water bath for 5 to 10 minutes, during which time some of the anthrax-specific antigens are dissolved if the tissue contains living or dead anthrax organisms. The material is filtered until a clear filtrate is obtained.

This is then layered in narrow tubes on undiluted anthrax-precipitating serum which has been proven by test.

At the line of contact, a white ring will form after a few minutes if anthrax antigens are present in the extract.

Plague

A rapid presumptive diagnosis on decayed tissues, especially of rats, may be made by applying the same principles as outlined in the Ascoli test to the detection of plague.

Procedure (Kolmer and Boerner):

One part of finely divided tissue is mixed with 5 to 10 parts of distilled water. Boil for 5 minutes and filter repeatedly through paper or asbestos wool with suction until perfectly clear. Using small tubes, set up the test as follows:

Place 0.5 ml. of high titer immune serum and carefully overlay with 0.5 ml. of filtrate.

Set up a duplicate using normal rabbit or horse serum (control).

Place 0.5 ml. immune serum and overlay with 0.5 ml. of normal saline solution (control).

Place 0.5 ml. of extract and overlay 0.5 ml. of normal saline solution (control).

A positive reaction shows a white ring of precipitate at the line of contact in the first tube in 5 minutes at 37°C., reaching a maximum after 2 hours. A negative reaction is not conclusive.

Trichinosis

The precipitin test is an accurate method of confirming a diagnosis of this condition in the presence of suspicious clinical symptoms. Although the finding of larvae in a biopsy specimen of muscle is more conclusive, such specimens are positive in only about 50 per cent of clinical cases. The antigen used for the precipitin test is prepared by making an extract of larvae digested out of the muscles of infected guinea pigs, using normal saline solution for the extraction. The antigen is used at a dilution of 1:20 or 1:100.

The precipitin test is performed in a small test tube (5 to 7 mm. diameter) by overlaying 0.5 ml. of patient's serum with an equal quantity of antigen (1:20 or 1:100). Mixing should be avoided. The tube

is incubated at 37°C. for one hour. A positive test is indicated by a white ring at the point of contact. A control tube using the patient's serum and normal saline should be prepared for comparison. This test occasionally becomes positive as early as the second week but usually not until the fifth week of infection. It persists for at least a year and is negative five to seven years after infection.

Echinococcus Disease

The precipitin reaction is one of several tests which may be used in the diagnosis of infection by *Taenia echinococcus*. The antigen is undiluted fluid from an echinococcus cyst. The test is performed by overlaying 0.5 ml. of the patient's serum in a small test tube, with an equal quantity of the cyst fluid. Incubate the tube at 37°C. for 30 minutes and observe the reaction. A positive test consists of the presence of a white ring of fine precipitate at the junction of the two fluid layers.

PRECIPITIN TESTS FOR THE IDENTIFICATION AND DIFFERENTIATION OF BLOOD STAINS AND OTHER SUBSTANCES*

The precipitin test for the detection of blood stains and other animal products occupies a valuable place in forensic medicine and is the only reliable and acknowledged method for the differentiation of proteins of different species.

Before setting up a precipitin test for the detection of blood, it is necessary to determine that the stain in question is blood, since other stains such as red paint, iron rust, etc. may have to be differentiated. If found to be blood it is then necessary to determine the species of origin.

The presence of blood may be verified by employing simple chemical tests such as Teichmann's, benzidine, quaiacum and spectroscopic. All these tests have their limitations.

The hemin test is reliable only when positive. The guaiacum and benzidine tests are reliable only when negative. When such tests are positive they carry no weight medico-legally. Spectroscopic absorption bands prove the presence of some derivitives of hemoglobin. If such derivatives exist only in traces absorption bands may not be discovered.

* Modified from Kolmer, J. A., and Boerner, Fred: Approved Laboratory Technic, 4th ed., D. Appleton Century Co., Inc., New York, 1945. Todd, James C., Sanford, Arthur H., and Wells, Benjamin B.: Clinical Diagnosis by Laboratory methods, 12th ed., W. B. Saunders Co., Philadelphia and London, 1953. Berger, H. I.: Blood, Clinical and Laboratory Diagnosis, Battle Co., St. Louis, 1930.

While chemical tests have no particular place in the chapter on serology, the tests for differentiating blood from iron rust and red paint are given below for convenience.

Differentiation of Blood from Iron Rust

Scrape or place small amount of material containing stain into a porcelain capsule containing HCl and heat gently.

If iron is present, the solution becomes yellow.

To potassium ferrocyanid add 1 drop of the yellow solution and the color turns blue.

To potassium sulphocyanid, add a drop of this yellow solution and a deep red color is the result.

Differentiation of Blood from Red Paint

The pigments in red paint are usually iron oxide and lead oxide.

Dried paint is insoluble in water but soluble in strong alkali solution or oil of turpentine.

Take some of this solution and allow to evaporate upon a watch glass.

Test for iron as previously described.

Lead is recognized by its vermillion color.

Chemical Tests for Lead Are:

1. The addition of potassium bichromate to any solution containing lead causes the precipitation of a yellow amorphous deposit, soluble in potassium hydroxide and strong HCl, but insoluble in acetic acid.
2. Hydrogen sulphide passed through lead solutions results in the precipitation of black lead sulphide.

Having determined by one or more of these tests that it is blood, it then becomes necessary to identify it as human blood or that of some other species. In order to do this, two reagents are necessary—antiserum and antigen. Antisera are prepared by the method outlined below. The specimen in question and other sera used in the test constitute the antigens.

Production of Anti-human Precipitins

Using aseptic precautions throughout, draw about 10 ml. of blood from the median basilic vein of several individuals. Place in sterile test tubes, allow to clot in a slanting position, rim upper part of clot with sterile inoculating needle and place in refrigerator overnight. Pipette clear serum from clot, pool, and place in suitable sterile bottles.

Immunize several rabbits by intravenous injections of human serum at 3-day intervals in the following amounts: 1, 2, 3, 4, 8 and 10 ml. or at 5-day intervals inject 2 doses of 8 ml. each, 2 of 5 ml., and 2 of 3 ml. To prevent anaphylactic shock the rabbits may be desensitized by injecting 0.2 ml. of the serum $\frac{1}{2}$ hour before injection of each of the later doses. Ten days after the last dose remove a small amount of blood from an ear vein and test the serum for precipitins by placing 0.2 ml. in a small test tube and carefully overlaying with 1 ml. of a 1:1000 dilution of human serum. Since the bulk of the precipitate comes from the antiserum, it can seldom be diluted whereas the antigen may react in dilutions exceeding 1:1,000,000. If a ring of precipitation occurs in a few minutes the fasting animal may be bled from the heart aseptically into sterile centrifuge tubes. After clotting has occurred, gently separate the clot and centrifuge to remove all corpuscles. Transfer the serum to sterile containers and to each 19 ml. add 1 ml. of 1:500 metaphen solution for preservation. For use the serum must be crystal clear for accurate results and this may require centrifugalization or filtration. Using aseptic precaution, place in sterile ampoules, label stating nature, type, titer (i.e., 0.2 ml. ppt. 1.0 ml. 1:1000 antigen), date and name of individual by whom prepared.

If the preliminary test is negative give the animal 3 additional doses of serum intraperitoneally in the following amounts: 10, 15 and 20 ml. Test again 10 days after the last dose.

For medico-legal work it is advisable to prepare similar precipitins for the sera of the commoner domestic animals as the hog, sheep, cow, chicken, dog, cat, etc. These are prepared in a manner similar to that employed for preparing anti-human precipitins.

Preparation of Unknown Material:

If the stain is on clothing or paper, a portion should be completely removed and cut into fine shreds, using forceps and scissors, not the fingers, and should be placed in a test tube of normal saline and shaken for 5 minutes until a lasting foam is obtained in the solution. Add more specimen or saline accordingly. This indicates an approximate dilution of 1:1000. If the stain is upon wood, metal, or glass, the stain should be completely scraped off with a *clean*, preferably sterile, knife, treated in a manner similar to stains on cloth, etc. An unstained portion of clothing, etc., containing the suspected material, should be extracted with saline to act as a further control on the technic in order to show that this does not give a reaction. It is sometimes necessary

to leave old specimens in the refrigerator 24 hours or longer. The solution should be perfectly clear or made so by filtration through a Seitz filter if necessary and should meet the following specifications:

1. Should be almost colorless by transmitted light.
2. It should give only a slight cloudiness, indicating a small amount of protein, when tested by the heat and acetic or nitric acid ring test.
3. It should foam freely on shaking.
4. It should be neither strongly acid or alkaline to litmus.

Neutralization may be accompanied by addition of a very weak solution of sodium hydroxide or hydrochloric acid.

If tests are negative for human blood other tests may be conducted with anti-chicken, beef, dog, cat and other anti-sera. In medico-legal cases it is always advisable to conduct these even though positive reactions are observed with anti-human serum.

Similar tests may be conducted for the detection of meat adulteration, identification of bones, milk, semen, etc. The technic for these tests may be found in "Infection Immunity and Biologic Therapy" by Kolmer, third edition, 1924—W. B. Saunders Company.

Method

Arrange a series of 7 small test tubes and charge them as follows, placing the second fluid beneath the first by means of a capillary pipette.

Tube No. 1—0.5 ml. of unknown protein solution dil. 1:1000 + 0.1 ml. of the immune serum.

Tube No. 2—0.5 ml. of unknown protein solution dil. 1:1000 + 0.1 ml. normal rabbit serum.

Tube No. 3—0.5 ml. physiologic saline + 0.1 ml. of the immune serum.

Tube No. 4—0.5 ml. of 1:1000 dilution known serum of the species suspected to be present in unknown material + 0.1 ml. of the immune serum.

Tube No. 5—0.5 ml. of 1:1000 dilution of serum of a species different from that suspected + 0.1 ml. of the immune serum.

Tube No. 6—0.5 ml. of the unknown solution alone.

Tube No. 7—If the solution of unknown protein was made from a stain on cloth, leather or other material, a tube should be set up containing 0.5 ml. of a saline extract of the material + 0.1 ml. of the immune serum.

Conclusions to Be Drawn from the Above:

If the first and fourth tubes give a definitely positive reaction, indicated by a distinct whitish ring at the zone of contact of the two fluids, and all the other tubes give a negative reaction, the presence of protein of the suspected species tested for is established. When only a limited amount of material is available the test may be made in pointed capillary tubes of about 3 mm. inside diameter.

Important Steps in Testing for Blood Proteins:

In medico-legal cases, no tests for blood should be performed without noting the following points:

1. The exact time and exact part of the clothing or other object from which the specimen was taken.
2. If the blood is to be scraped from an instrument, describe fully.
3. Note shape and size and direction of all stains. The small part of the spatter points to the oposite direction of the hemorrhage.
4. Specimens must have been properly sealed; the sender's name recorded; date and time of delivery noted, and seal must be unbroken.
5. Examine only a small portion of the specimen. Keep the remainder for corroborative evidence.
6. Blood appearing on one's person may be there accidentally by reason of one's occupation, e.g., butchers. Or, in women, menstrual blood must be considered.
7. Time not only alters the appearance of blood, but also influences the tests for blood.
8. Blood stains upon a knife or other metal must be distinguished from rust or lemon stains. All weapons should be examined immediately.
9. Blood stiffens all fabrics. Under a hand microscope, blood stains on linen give a dark crimson reflection, are glossy, smooth or reddish brown.
10. The action of various substances such as mortar, brick, lime, strong acids or alkalis, leather, chemicals in wall paper, starched clothing, etc., may so change the blood or impair its reactivity to certain tests so that no definite conclusions can be drawn.

Interpretation of Results

While the precipitin reaction is clearly specific and its usefulness in medico-legal work is well recognized, it must be remembered that the

reaction does not prove the presence of blood but only that of a protein of the species indicated. Where doubt arises, and proteins of very closely related species are involved such as those of man and monkey, sheep and goat, it can practically always be removed by the use of adequate controls. Alcohol, formaldehyde, strong acids and alkalies, heat and decomposition may reduce or destroy the power of protein to react with precipitin. Blood in the fluid stage is less resistant to these conditions than dry blood.

Other Precipitin Tests

For precipitin reactions applied to the diagnosis of syphilis see Chapter 19, Syphilis Serology.

COMPLEMENT FIXATION TESTS

The complement fixation test is primarily thought of as being applied to the serologic diagnosis of syphilis (see Chapter 19), yet the principles of this test find wide application in diagnosis of certain diseases due to bacteria, rickettsiae, a few of mycotic and viral origin and a variety of those caused by animal parasites. In fact, Widal and Lesourd applied the Bordet-Gengou reaction to the diagnosis of typhoid fever (1901) before Wassermann, Neisser and Bruck applied it in their epoch making work on syphilis (1906). Using an emulsion of typhoid bacilli as antigen and sera of typhoid cases, they found that a positive reaction occurred more frequently and earlier than with the agglutination test. Furthermore, it is extremely valuable in its application to forensic medicine for the detection and species identification of blood stains and of meat adulteration.

To whatever diagnostic use they are applied, all complement-fixation tests are alike in principle, each differing primarily in the antigen used and in varying modifications of the technic employed.

Glanders

In humans the test is less apt to be successful in acute than in chronic forms, as the course of the disease is often short in the former, and sufficient antibody formation is lacking. The procedure is most useful in testing horses. The antigen consists of killed cultures.

Gonococcal Infections

Due to lack of uniformity in procedure, the test alone is not absolutely reliable. Negative results are sometimes obtained in the pres-

ence of positive findings and positive results are sometimes obtained with no history of infection and in the face of negative bacteriological tests. It is most useful as a diagnostic procedure in suspected gonorrheal arthritis, pelvic inflammatory diseases, and chronic prostatitis. For detailed method of the test, see Chapter 18, The Diagnosis of Venereal Lesions.

Hemophilus Pertussis

While the use of this test has been reported by a number of investigators, it has not been developed sufficiently as a diagnostic procedure.

Rickettsial Diseases

The complement fixation test may be used as a means of differentiating the following rickettsial diseases:

Epidemic from endemic typhus	The test becomes positive about the 8th day and increases in titer and remains positive for years after the attack.
Typhus from spotted fever (both forms of typhus from spotted fever)	The test usually becomes positive about the 10th day, remaining positive for years after convalescence.
Q Fever	Test becomes positive about the 2nd week.

Specific antigens for differentiating the rickettsial diseases indicated above are usually prepared by growing rickettsiae in the yolk sac of developing chick embryos by the Cox method. Unless one is doing tissue culture work, antigens are best bought from dealers in biologic preparations or the tests performed by the National Institute of Health at Bethesda, Maryland. Laboratories of some state health departments also are prepared to perform these tests. Detailed accounts of the tests are to be found in the writings of Cox, Bengtson, Plotz and Wertman.

Epidemic Encephalitis

In the hands of Casals and Palacios, the complement fixation test has proved useful in distinguishing between simultaneously occurring epidemics of encephalitis and of poliomyelitis.

The antigen used is the supernate preserved in methiolate, prepared from a centrifuged emulsion of infected mouse brain in ten times its

weight of physiologic saline. Detailed description of the preparation of the antigen and the test may be found in the papers of the authors

Amebiasis

Craig, in 1927, pointed out that alcoholic extracts of cultures of *Endamoeba histolytica* possessed marked complement-binding power when used as antigen in complement fixation tests of individuals infected with this parasite.

Of 689 cases checked for the parasite, 84 gave a positive complement fixation reaction and 92 per cent of these were shown to have the parasite in the feces. Sera of the remaining 605 cases gave negative results. The parasite was found in 5, or 0.8 per cent of these cases. One of these cases was a severe acute amebic dysentery; one was amebic abscess of the liver; one was symptomless while the other two showed gastrointestinal symptoms that were suspicious. From these findings, it is concluded that persons infected with this parasite do not all give a positive reaction, but the percentage of negative results is apparently very small.

The author concludes that the complement fixation test applied to the diagnosis of amebiasis finds its greatest value when a competent protozoologist or an individual competent to recognize the parasite in feces is not available; or for differentiating various ameba of the human intestine and in checking diagnoses made by untrained or improperly trained laboratory workers. This test may prove useful in the detection of carriers.

Detailed description of the preparation of the antigen and the test may be found in the original writing and also in Gradwohl.

Chagas' Disease

The use of the complement fixation test for diagnosis of Chagas' disease, as developed by Kelser, is simple and reliable. Diagnosis of Chagas' disease is difficult and not easily accomplished by usual laboratory methods, and this test becomes a useful and valuable agent, not only in the diagnosis of the disease but in the identification of carriers.

According to the author, no cross reactions occur with positive syphilitic serum, but sufficient work has not been done to determine whether cross reactions will occur with sera from other types of trypanosomiasis.

The antigen is easily prepared and consists of an extract of cultures of the organism grown on Kelser's medium. Detailed description of

the preparation of the antigen and the test may be found in the writings of the author.

Malaria

The complement fixation test has not reached the perfection of diagnostic value in malarial infections.

Hydatid Cysts

The complement fixation test for the diagnosis of hydatid cysts is not specific but is a group reaction. Antigens may be prepared from hydatid fluid, an extract of *Taenia saginata*, *Fasciola hepatica* and the fluid from *Cysticercus tenuicollis* but are best purchased on the market from manufacturers of biologics. The reaction may sometimes be positive in infestation with *Taenia saginata* or *Fasciola hepatica*. In hydatid diseases, the serum may give negative results two months after removal of the hydatid cyst.

While this test yields better results than precipitin tests, it is not as reliable as the intradermal reaction in which the percentage of positive reactions is much higher, approximately 87 per cent. The latter test is not only more simple to perform but gives positive results long after the hydatid cyst has been removed.

Since antigens may yield cross complement fixation reactions with sera containing large amounts of syphilitic antibody, it is necessary to parallel this test with a complement fixation test for syphilis. When a serum gives a positive complement fixation test for syphilis, great care should be observed in interpreting the results positive for echinococcus.

Trichinosis

This test, as developed by Witebsky, Wels, and Heide, is sensitive and specific. The antigen consists of boiled watery extract of dried larvae. According to the authors, the test can be carried out as a quantitative procedure and the antibody titer can be observed over a period of time.

Preparation of the antigen consists primarily in infesting rats by feeding trichinous meat. After 5 or 6 weeks, the rats are killed, skinned and eviscerated. The meat is minced, ground and digested with pepsin and hydrochloric acid solution. This liberates the larvae from the cyst, after which they are washed by centrifuging with saline and dried in a desiccator. This constitutes the material from which the antigen is prepared by watery extraction. Full details of the entire procedure may be found in the paper by the authors.

Other conditions in which the complement fixation test may be employed as a diagnostic aid are in parasitic infestations due to *Schistosoma, Leishmania, Borrelia, Fasciola hepatica* and *Paragonimus westermanii*, and also in certain fungus infections such as blastomycosis (pulmonic and systemic), coccidiomycosis, moniliasis and sportrichosis.

In addition to the bacterial diseases already considered, complement fixation tests may be used in typhoid, paratyphoid, plague, bacillary dysentery and undulant fevers, and in tuberculosis—particularly pleural exudates and cerebrospinal fluid in the diagnosis of tuberculous pleuritis and meningitis. With few exceptions, however, fixation tests as yet offer little advantage over the laboratory methods commonly used as diagnostic aids in these conditions.

Complement Fixation and Other Serological Tests in Forensic Medicine

These tests as applied to forensic medicine hold much in the balance and are of exceptional value in the hands of the skilled and experienced worker. They may be used in identifying human blood, semen, saliva, bones and other tissues as well as in detecting adulteration of meats and milk.

Having exceptional rather than general clinical application, technical methods are not included here but are to be found in special treatises.

Each type of serologic test has its special place in forensic medicine, the choice of which depends upon its specific need.

SKIN TESTS

Testing for Sensitivity to Serum:

Before the administration of antisera, particularly that containing horse serum, it is necessary to determine whether the individual is sensitive. Persons who have previously had a serum injection or those who give a history of allergy in any form may show the "accelerated" type of reaction following administration of serum. Reactions of this type usually occur within a few minutes, a few hours, or up to 3 or 4 days following the injection. Immediate manifestations may develop in severity. There is usually a generalized erythema with urticarial swelling. The pulse becomes quick and the blood pressure falls. Syncope or unconsciousness may result. The mucus membranes may be involved, with the production of sneezing, lacrimation, dyspnea and wheezing. There is a possibility of "allergic shock" which may terminate fatally. Therefore, before administering antiserum, the following important questions should be asked the patient:

Is there hay fever, asthma or eczema in the patient's family?

Is the patient subject to allergic manifestations, such as hay fever vasomotor rhinitis, or drug idiosyncracies?

Has he ever been around horses? If so, does such exposure produce sneezing, blocked nose, itching of the eyes, coughing, wheezing with difficulty in breathing?

Has he had a previous serum injection such as "tetanus shots"? How long ago?

All this information is pertinent to the administration of the therapeutic serum.

Tests for sensitivity should be made on every patient on whom serum administration is indicated, and a positive history, coupled with the results of such tests, becomes a valuable guide on which administration of serum is based.

Methods:

1. The intracutaneous test which consists in the injection of 0.02 ml. of a 1:10 dilution of therapeutic serum. A positive reaction usually occurs promptly or within 30 minutes and consists of a urticarial swelling with central blanching, peripheral erythema and itching.

2. The conjunctival or ophthalmic test which consists in instilling a drop of diluted serum into the lower conjunctival section. First, a 1:100 serum dilution is used and if this is negative, then test with a 1:10 dilution. Positive reactions usually develop within 2 to 10 minutes and are characterized by redness, due to injection of the conjunctival vessels, itching, or the sensation of a foreign body, and lacrimation. Marked reactions may result in swelling of the conjunctiva or of the lower lids, persisting for twenty-four to forty-eight hours. If properly carried out, the test is harmless and may be controlled with one or two drops of 1:1,000 epinephrine. Glycerinated solutions can be used in the ophthalmic test.

Interpretation: When both intradermal and ophthalamic tests are positive, a higher degree of constitutional or general tissue sensitivity exists than when the intradermal test alone is positive. Therefore, one should proceed with great caution before attempting to administer anti-serum.

If the patient is subject to asthma due to horse dander and if both of the above tests are positive, administration of horse serum is likely to result in severe reactions and its use is contraindicated. For more detailed procedure of serum therapy in the presence of demonstrated sensitivity see Fantus (bibliography) and Vaughn-Black.

Tuberculin Tests

1. *Intracutaneous Method (Mantoux).* The antigen used may be either Old Tuberculin (O.T.) or the new Purified Protein Derivative of tuberculin (P.P.D.) which is the active principle of O.T. The latter is recommended because of its uniformity of dosage. P.P.D. is prepared in tablets of two strengths containing respectively 0.0002 and 0.05 mg. of the active substance. When dissolved in 1.0 ml. of buffered saline, 0.1 ml. of solution contains 0.00002 mg. and 0.005 mg. of tuberculin.

Technic: The test is made by injecting 0.1 ml. of the first strength solution (containing 0.00002 mg.) into the skin on the flexor surface of the forearm. The skin is cleansed with alcohol and a tuberculin syringe and 26 gauge, half-inch needle used. Care must be taken that the material be injected into the skin. Read the test in 48 hours and if negative, inject intradermally 0.1 ml. of the second strength (containing 0.005 mg.) into the opposite arm. Read in 48 hours.

Interpretation: A positive reaction is one which shows redness and edema after 48 hours or more (Plate 11.II). Edema is more important than redness and if absent the test is negative. The following criteria are recommended by the National Tuberculosis Association:

One-plus (+)—Redness and edema 5 to 10 mm. in diameter.
Two-plus (++)—Redness and edema 10 to 20 mm. in diameter.
Three-plus (+++)—Marked redness and edema exceeding 20 mm. in diameter.
Four-plus (++++)—Redness, edema and central necrosis.
Doubtful (±)—Slight redness and a trace of edema 5 mm. or less in diameter.

When reading the test, the arm should be slightly flexed at the elbow. Look across the arm instead of down at it. Palpate the area to detect induration or edema.

A positive tuberculin test means that tubercle bacilli have in the past or are at present infecting the tissues. It does not indicate necessarily that active clinical tuberculosis is present or has ever existed. Negative tests may be seen in acute miliary tuberculosis, possibly due to mass hyposensitization. Occasionally, a positive test may become negative after a variable period of time.

2. *Patch Method (Vollmer).* The test material consists of a strip of adhesive tape containing two test squares with a control square between. The test squares are filter paper saturated with tuberculin produced on a synthetic medium. The dose of antigen corresponds to one midway beween that of first and second strength O.T. (0.1 mg.).

Technic: An area of skin between the scapuli or on the flexor surface of the forearm is cleaned with acetone. The adhesive strip containing the test patches is applied. Instruct the patient to keep the adhesive dry. The test is removed at 48 hours and the reaction read 48 hours later (96 hours after application of test).

Interpretation: A positive reaction is indicated by a red, edematous square which may show vesicular or follicular elevations. The control square appears pale.

This method of testing has found favor in pediatric practice since it is simple, quick of application and painless. In infants and young children, excellent correlation with the intradermal method has been reported. With adults correlation with P.P.D. testing has been poor. Possibly decreased skin sensitivity may account for this.

TESTS FOR HYPERSENSITIVITY TO ANIMAL PARASITES

Skin testing has been used in the diagnosis of many types of parasite infestations, but most satisfactorily in trichinosis and hydatid disease (echinococcus). Testing with the more common types of intestinal parasites has met with indifferent success.

Skin Test For Trichinosis

The antigen used is prepared by making an extract of larvae digested out from the muscles of infected guinea pigs, using physiologic saline or Coca's solution for the extractions and making a final dilution of 1:10,000.*

The test is performed by injecting 0.1 ml. of the antigen (1:10,000) intracutaneously on the ventral aspect of the forearm. A control test using normal saline should be performed on the other arm. Two types of reaction are possible, the immediate reaction and the delayed reaction. The immediate reaction consists of a blanched wheal one centimeter or more in diameter, often with pseudopods and surrounded by a zone of erythema which may reach several centimeters in diameter. The reaction appears within 10 minutes, reaches its maximum within 30 minutes, and may disappear within an hour. This reaction usually becomes positive during the second week of the disease and usually remains positive for years. The delayed reaction consists of redness and swelling appearing about 24 hours after the test is performed. This reaction may be positive by the second or fourth day of illness but is negative after about

* Antigen prepared with physiologic saline may be obtained from the National Institute of Health, Washington, D. C.

the first week. Non-specific reactions are sometimes obtained if a strong concentration (1:100) of antigen is employed but never with a 1:10,000 dilution. For interpretation see Chapter 14, Medical Parasitology.

Hydatid Disease (Echinococcus Infection)

Casoni devised an intradermal test for hydatid disease which is said to be about 95 per cent accurate, but remains positive throughout the patient's life, even following a cure.

The antigen consists of a sterile hydatid fluid from an uncomplicated echinococcus cyst, prepared by passing the material through a Berkefeld or Seitz filter. Fluid may be obtained from infected human beings, sheep, cattle or hogs. After preparation, it is tested for sterility, placed in ampules, which, when kept in the refrigerator, are usable up to six months.*

The test is carried out by injecting 0.25 ml. of the sterile antigen into the skin of the flexor surface of the forearm, forming a good sized wheal; a saline control of the same volume should be used on the opposite arm.

There are two types of reaction:

Immediate, when the wheal rapidly increases in size, becoming surrounded by marked erythema and pseudopodia, reaching its maximum in about one-half hour and then disappearing. The positive reaction should be a wheal of at least 2 to 2.5 centimeters in diameter.

Delayed, when the erythema and induration appear in from 18 to 25 hours, in which there is edema and infiltration 4 to 6 centimeters surrounding the site of injection.

The wheal is usually surrounded by a zone of hyperemia but this is not as important as the size of the wheal and the presence of pseudopodia. The test is of value preoperatively. However, in postoperative cases, as indicated above, reactions may be observed over long periods of time. Positive reactions are not absolutely indicative of hydatid diseases since false positives or pseudoreactions may occur from hypersensitiveness to sheep serum in the antigen, from trauma, and from other cestode infestation.

The test may be negative if the cyst possesses no antigenic properties, or if the cyst has died or been evacuated. A negative reaction indicates absence of infection in about 95 per cent of the cases.

Culbertson and Rose have used antigens made from other helminths and have noted that these serve almost as effectively as that prepared

* Antigen for testing may be obtained from Sharp & Dohme, Philadelphia, Pa.

from the echinococcus. This is of diagnostic import in interpreting the test which is evidently not specific for *E. granulosus*.

Results may be checked with the precipitin and complement fixation tests. However, the skin test is said to be much more satisfactory than the complement fixation test. Lemaire and Theiodet in 1926 obtained the following percentage of positive reactions in individuals having hydatid cysts:

Precipitin reaction	17 per cent
Complement fixation	17 per cent
Intradermal reaction	87 per cent

TESTS FOR ANTIBODY FORMATION

The underlying reaction in these tests is in no way related to hypersensitivity but the methods employed are the same so they are included here for convenience. The principle involved is that of neutralization of toxin by specific antitoxin. Injection of the toxin will produce an area of inflammation unless it is neutralized by a specific antitoxin, thus a positive skin reaction indicates an absence of antitoxin or lack of immunity to the disease in question.

The Dick Test

The test is used to prove the development of immunity to scarlet fever after active immunization, and to determine the susceptibility to individuals who may be exposed to the disease. The test material is a specially standardized dilute solution of scarlet fever toxin of such strength that 0.1 ml. contains 1 skin test dose which is the quantity of toxin that will induce a reaction when injected intracutaneously into persons of known susceptibility to the toxin. The test provides a simple clinical measure for determining susceptibility to scarlet fever and deciding upon the advisability of administering antitoxin for prophylaxis or toxin for immunization.

Technic: The test is made by injecting intradermally, on the flexor surface of the forearm, exactly 0.1 ml. of the skin test solution of toxin. The reaction is read 20 to 24 hours later.

Observations: Slightly positive reactions consist of a faint red area less than 1 cm. in diameter, without swelling of the skin, and without tenderness.

Positive reactions are 1 to 3 cm. in diameter, bright red, with some swelling of the skin and occasionally slight tenderness.

Strongly positive reactions are more than 3 cm. in one or both diam-

eters, intensely red, with marked swelling of the skin, and usually some tenderness. These strongly positive reactions are frequently from 5 to 7 cm. in diameter. The swelling shows a partially raised edge that extends beyond the reddened area.

Control: That the positive reactions are not caused by foreign protein in the culture medium may be shown by the simultaneous infection in a different area with boiled toxin.

Interpretation: If there is no antitoxin in the blood or tissues of the individual, or if antitoxin is present in very small amounts insufficient for protection, a positive reaction will appear within 24 hours. The slightest flush or reddening, no matter how faint the color, constitutes a positive reaction if it measures as much as 1 cm. in diameter. The Dick test reaction is more transient than the Schick reaction and is often faded if observed later than 24 hours after the test has been made. There is no induration characteristic of the Schick Test.

If negative reactions show either no change or a slight redness less than 1 cm. in diameter, it is evident that the individual is immune to scarlet fever (Plate 11.II).

Schultz-Charlton Blanching Test

This test is of clinical value as an aid in differentiating scarlet fever rashes from similar rashes occurring in Rubella and various drug allergies (quinine, salicylates, etc.).

Technic:

The test is carried out by intracutaneous injection of 0.5 ml. of convalescent scarlet fever serum or 0.1 ml. of a potent scarlet fever antitoxin in the center of a large area where the rash is brightest, preferably on the abdomen or chest. A positive reaction consists of blanching of the rash in a zone surrounding the central red spot where the injection was made. Observation for the reaction is made 18 to 24 hours following injection. Rashes due to Rubella (German measles) and drug allergies will not blanch if the test is made with scarlet fever antitoxin.

If convalescent scarlet fever serum is used, some blanching of non-scarlatinal rashes due to infectious diseases may occur if the serum happens to contain sufficient antibodies for them. It is, therefore, best to use standard scarlet fever antitoxin.

Schick Test

It has been shown by Roemer and others that the blood of normal individuals, in a good many instances, contains a small amount of diph-

theria antitoxin. The presence of such antitoxins in individuals may be demonstrated by means of the Schick test or Schick reaction.

Standard diphtheria toxin is diluted in normal salt solution so that 0.1 ml. contains 1/50 M.L.D. for a guinea pig. (Some test outfits call for this dose to be contained in 0.2 ml.) This is injected intracutaneously. If the blood of the individual receiving such injection contains less than 1/30 unit of antitoxin per ml., a positive reaction appears in 24 to 36 hours. The reaction is evidenced by the formation of slight infiltration of the skin surrounded by a red areola 1 to 2 cm. in diameter. A negative reaction indicates that sufficient natural antitoxin is present in the blood of the individual to protect against diphtheria. With individuals giving a positive reaction, it has been found advisable to administer prophylactic doses of diphtheria, toxin-antitoxin mixture to adults; toxoid to children.

Technic: 0.1 ml. of test toxin is injected intradermally on the cleansed skin of the flexor surface of the forearm below the elbow. Because a certain number of individuals, varying between 5 and 20 per cent, give a pseudo or false reaction caused by the proteins of the diphtheria bacilli used in producing the toxin, it is necessary to inject control fluid in a like manner in the opposite arm. It is usually customary to inject the test toxin in the right arm and the control fluid in the left. Successful intradermal injection is indicated by the presence of a wheal which forms at the site of injection. The control fluid is Schick toxin which has been heated to 80°C. for a period of ten minues. Heating destroys the toxin but does not alter the bacterial protein content. Pseudo reactions are more likely to appear in older children and adults, especially those who have been vaccinated against diphtheria. The control may be omitted in children under six who have not been previously immunized.

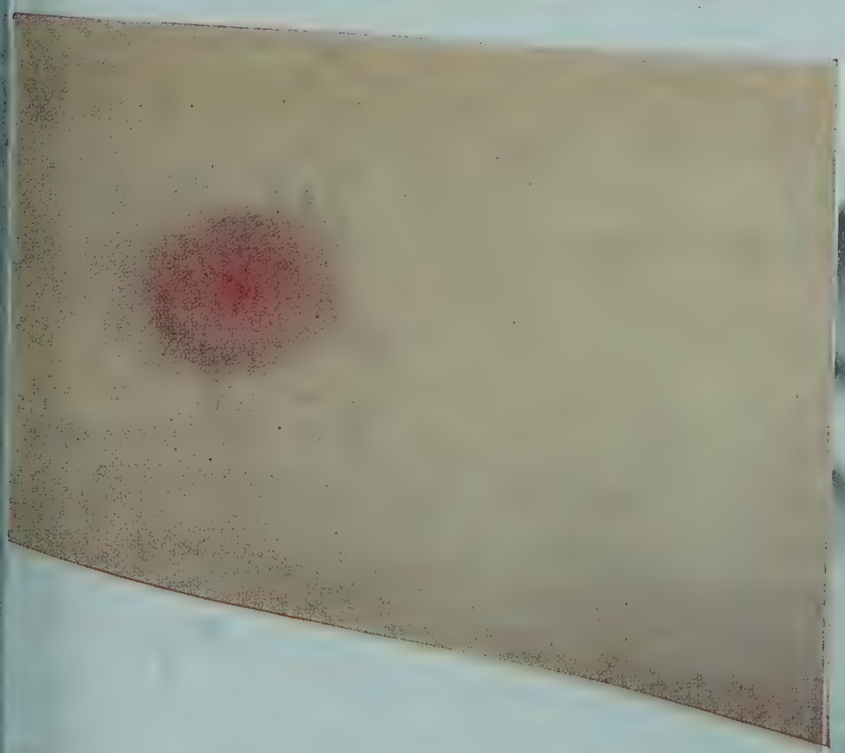
The Negative Reaction. Following the injection, no signs are present on either arm except the slight and fleeting mark incident to the insertion of the needle. If the test has been properly done with the proper toxin dilution, the absence of reaction indicates that the individual has as

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PLATE 11.II. A. Positive Schick test. Photographed at 72 hours. The two needle punctures visible distal to the reaction are control and Dick tests; both were negative in this case. B. Positive Dick test. Photographed 24 hours after intradermal injection of one skin test dose of Scarlet Fever Streptococcus Toxin. C. Positive tuberculin test. Photographed 72 hours after intradermal injection of 0.1 cc. of the first test strength of tuberculin, P.P.D. (Purified Protein Derivative). D. Positive Schick test (72 hours). A small bleb appeared in the center of this reaction, but had been pricked by the patient shortly before the reaction was photographed. (From photographs made at the Children's Hospital, Detroit, Mich., through the courtesy of Dr. Thomas B. Cooley.)









D

much as 1/30 unit of antitoxin per ml. of serum and is immune to diphtheria. Recent investigation has shown that as little as 1/100–1/250 unit per cc. is regarded as sufficient to confer immunity.

The Positive Reaction. A positive reaction begins to appear on the right arm (toxin dilution injection) in 24 to 36 hours and is characterized by a circumscribed area of redness and slight infiltration which measures 1 to 2 centimeters in diameter. It develops gradually, reaches its greatest intensity on or about the fourth day, then fades very slowly, leaving a scaly, brownish pigmented spot which eventually disappears. There is no reaction at the site of the injection of the heated toxin dilution. The positive result of the test signifies that the individual possesses little or no antitoxin in the blood, and therefore may contract the disease (Plate 11.II).

The Pseudo Reaction. In some individuals, particularly in adults, a reaction develops which may be confused with a positive reaction. Owing to a hypersensitiveness of some persons to the protein of the diphtheria bacillus present in the toxin, a local reaction may appear at the point of injection. This reaction is differentiated from the true positive reaction by means of the injection of the heated toxin dilution. If a reaction develops at the same time at the sites of both injections, runs a similar course, reaching a maximum of intensity on the third day and then fading, the reaction is classed as a pseudo reaction and the individual is considered hypersensitive to the protein of the diphtheria bacillus, but is immune to diphtheria.

The Combined Reaction. If a combined reaction is present, the redness and infiltration at the site of the "Toxin Dilution" injection will be more marked at the end of twenty-four hours than at the site of the "Heated Toxin Dilution" injection. At seventy-two hours, the positive reaction will be quite distinct, while the control test will show only a blotchy area of pigmentation representing the pseudo reaction elements of the test. If the test is positive, the reaction at the end of 96 hours will be much more marked at the site of the unheated toxin injection.

Interpretation: The negative and pseudo reactions indicate immunity, the positive combined reactions, susceptibility to diphtheria. A short experience in reading the reactions will suffice to enable one to make a correct interpretation of the results. (See colored plates, differentiating the different types of reactions, in the Diphtheria Monograph of British Med. Research Comm.)

In interpreting the different reactions, it must be remembered that the "toxin" injected in the Schick test is not simply a solution of pure

diphtheria toxin. The Schick toxin is a bacteria-free filtrate of a culture of diphtheria bacilli properly diluted to contain the desired concentration of toxin. While the bacilli have been removed by filtration, the materials include, in addition to the true toxin, all the soluble products of growth of the bacteria together with a considerable amount of bacterial protein which has been liberated by autolysis of the diphtheria bacilli. The broth culture medium itself is also present. It is the complex mixture of substances other than the true toxin, which complicates the interpretation of the reactions.

The Moloney Test for Detecting Sensitivity to Diphtheria Toxoid

Due to bacterial hypersensitiveness or to a general allergic state of the individual, older children and some adults may show a severe local and general reaction following the use of plain and, in some instances, alum-precipitated diphtheria toxoid. Such sensitivity can be detected by use of the Moloney test.

Technic: 0.1 ml. of a 1:20 dilution of plain (not alum-precipitated) toxoid is injected intracutaneously on the flexor surface of the forearm.

A positive reaction consists of a red area of not less than 12 millimeters in diameter occurring in 12 to 24 hours. This test gives definite and useful information when used for ascertaining the presence or absence of hypersensitiveness to toxoid, preliminary to its administration in adults and in children over 12 years of age. In case of positive reactions, the total dosage is usually split into 3 or 4 injections at intervals of three weeks, incorporating epinephrine with each dose.

Smallpox Vaccination

Vaccinia (smallpox virus) is secured in calf lymph and used as a protective measure against smallpox. Choose the site for vaccination, usually the skin over the insertion of the deltoid muscle on the left arm, and clean thoroughly with acetone or alcohol and allow to dry. Do not use iodine or other disinfectants. There are many methods of vaccination, the most recent and probably the best being the multiple puncture method. The virus is squeezed out in a drop on the cleansed skin surface, and through this drop, the skin is pricked in a low tangential manner. The side of the needle point is pressed into the drop about 30 times in 5 seconds. No signs of bleeding should occur. After vaccination, wipe off drop of vaccine with piece of sterile gauze. Then allow to dry. Do not apply a vaccination shield. This acts as an incubator and sometimes leads to infection. In young children where there is danger

of handling or scratching, a layer or two of sterile gauze may be gently applied and bound with small strips of adhesive. Severe reactions may be covered in the same manner.

Vaccination against smallpox should be performed on children, first, between 2 and 6 months of age, and then repeated at 5 to 7 years and at 15 years.

The types of reaction following vaccination are illustrated diagrammatically in Fig. 11.12.

Description of Various Types of Reaction Following Vaccination

The four types of reaction which may follow vaccination are briefly as follows:

1. *Vaccinia or Primary Reaction:* Seen in those never previously vaccinated or whose immunity from previous vaccinia or smallpox has completely disappeared. The papule appears on the third to the fifth day and the vesicle reaches its greatest development on the eighth or tenth day, or later.

2. *Vacciniod or Accelerated Reaction:* Seen in those retaining partial immunity from a previous attack of smallpox or a previous vaccination. The papule appears on the third or fourth day, with vesicle formation by the fifth day. Pustulation is frequent. The height of the reaction occurs on the sixth or seventh day, after which it subsides rapidly.

3. *Reaction of Immunity or Immediate Reaction:* Seen in persons possessing a high degree of immunity. A papule appears within forty-eight hours and subsides without forming a vesicle.

4. If none of the above reactions occurs or if there is delayed formation of a small papule after the third day, without further development, this is a negative reaction. The person should be revaccinated. Failures are nearly always due to deteriorated or impotent vaccine, or to faulty technic.

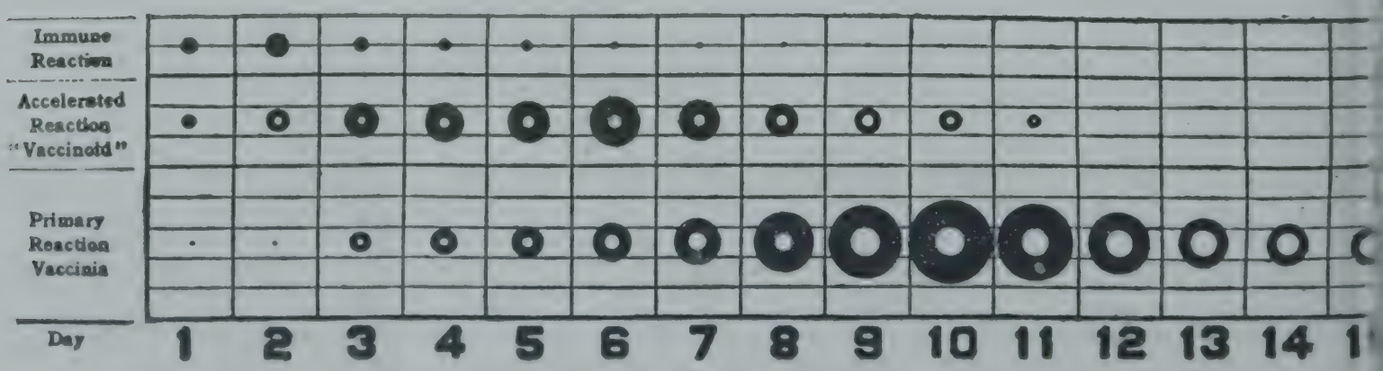


FIG. 11.12. Diagrammatic illustration of types of reaction following vaccination. (After Zinsser and Bayne-Jones: Textbook of Bacteriology, Appleton-Century Co.)

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CHAPTER 12

MEDICAL BACTERIOLOGY

By Albert Milzer

Bacteriologic Examination of Blood

BLOOD CULTURES

General Considerations

Blood culture is employed for diagnosis in determining the presence and nature of bacteria in the blood stream of the patient. It is also a valuable clinical guide to prognosis and for determining results of treatment. It cannot be overemphasized that preliminary blood cultures should be taken before antibiotic therapy is instituted (see Chapter 9). Blood culture is indicated in many conditions where the presence of bacteria (bacteremia) or bacteria and their poisonous products (septicemia) are suspected. The increase of bacteria in the blood is usually the result of greater invasion from a focus of infection rather than multiplication in the blood stream per se. Pyemia occurs if secondary foci of suppuration and multiple abscesses are formed. In bacteremia blood culture is widely employed in suspected cases of typhoid and other enteric fevers, during the first week of infection, while it is almost routine in cases of septicemia due to generalized infection with Gram-positive cocci.

Clinically, bacteremias are of two types, continuous and intermittent (Bennett *et al.*, 1954). Continuous bacteremia is usually a sign of serious infection (Ottenberg, 1934). The simplest form is that which results from the multiplication of bacteria in an area of the body bathed by the circulating blood, i.e. bacterial endocarditis, infected patent ductus arteriosus or arteriovenous fistula. Bacteria are constantly present in the blood during the onset of the typhoid group of infections, pneumococcus pneumonia, brucellosis, cerebrospinal meningitis and may occur in other bacterial infections. The prognosis is guarded if organism continue to be cultured from the blood later in the course of pneumonia (Dowling, 1948) and other infectious diseases. The prognosis becomes grave if they persist and their number increases unless effective specific therapy is available.

Many types of transient bacteremia are of little consequence and are usually the result of a sudden shower of bacteria into the blood stream. These usually follow manipulation of infected tissues or instrumentation

of the genito-urinary tract (Powers, 1936). Bacteremia has been observed to accompany such physiological conditions as normal labor and menstruation of apparently healthy women (Kulka, 1930). Several types of operative procedure may be accompanied by bacteremia. Various authors have reported bacteremia immediately after incision of an abscess, tonsillectomy and tooth extraction (Fisher *et al.*, 1941; Murray *et al.*, 1941). Reith *et al.* (1932) studied random blood cultures of 293 apparently normal subjects, of whom 278 were males, over a period of several months. One or more positive blood cultures were obtained in 65 normal subjects chiefly during the months of the year when upper respiratory tract infections were common.

When blood cultures are desired the bacteriologist should be informed of the nature of the suspected infection so that proper media and methods of culture may be selected.

The time when the patient's temperature is rising should be known in order that the specimen may be taken when the probability of obtaining organisms from the blood stream is greatest. Several authors (Kotin *et al.*, 1952; Weiss *et al.*, 1932) however, feel that blood for culture should be obtained during the hour preceding the expected rise in temperature in the patient with intermittent chills. They point out that this is important because the blood is often sterile at the onset of a rising temperature. Bennett *et al.*, (1954) believe that the proper schedule for endocarditis patients is a few blood cultures daily for several successive days rather than many cultures in a single day. On the other hand, in chronic meningococcemia many cultures may be necessary for isolation of the organism. Wells (1950) states that while most clinicians insist on the routine of taking blood for cultures at the peak of temperature, or in relation to chills, there seems to be no real advantage in this procedure.

Kolmer *et al.* (1951) emphasize the fact that more consideration should be given to the choice of vein in securing blood for culture. As a general rule, the median basilic vein of the arm is employed, but apparently this is not always a good routine procedure. It has been shown that in septicemia due to thrombophlebitis, blood taken from the vein directly draining the focus contains more organisms than venous blood removed from the arm because of less dispersion. In view of this, it would seem advisable to draw blood from a vein draining an infected area whenever feasible. It has been shown by Murray *et al.* (1940) that cultures of blood removed from the femoral artery have shown a higher incidence of positive results than duplicate cultures of venous blood. Beeson *et al.*

(1945) state that the balance of experimental and clinical evidence indicate that there is no removal of bacteria in the circulation of the extremities. Consequently, blood from the antecubital vein is as likely to contain organisms as blood from the brachial or femoral arteries. According to Wells (1950), more recent evidence also does not support the conclusion that arterial rather than venous blood is more likely to yield positive results. Culture of sternal marrow may show positive results when venous blood fails. This is especially true in typhoid after the first week of the disease and in brucellosis (Sacks *et al.*, 1940). Bone marrow aspiration is also an aid in the diagnosis of histoplasmosis (see Chapter 13). The superiority of the marrow culture in these diseases is probably due to the fact that there is a more abundant source of macrophages containing viable organisms in the bone marrow than is found in the peripheral blood.

Streptococci, usually members of the viridans group, or more rarely staphylococci can be recovered in a majority of cases of subacute bacterial endocarditis if cultures are made. On the other hand, hemolytic streptococci may cause a fulminating ulcerative endocarditis. Of the acute infectious diseases pneumonia and epidemic meningitis are associated with bacteremia in about half of the cases.

In cases of acute articular rheumatism, subacute bacterial endocarditis, puerperal sepsis and subacute focal infections, duplicate cultures should be made, one incubated aerobically, the other anaerobically. In cases of puerperal sepsis, suspicion should be directed toward the presence of hemolytic streptococci and *Cl. perfringens*.

Anaerobic cultures should be made in all suspected cases of postoperative septicemia in relation to hemolytic streptococci and the genus *Bacteroides*. For anaerobic culture a thioglycollate medium is preferable.

Blood cultures should be examined at daily intervals for four weeks before being discarded as negative.

Incubation in an atmosphere of 10 per cent CO_2 for primary isolation is necessary for some organisms such as *Brucella abortus*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Organisms recovered are further identified by the usual methods found in standard texts on bacteriology. Separate plating is resorted to if more than one species is present.

In cases of otitis media, blood cultures are almost always negative. When the inflammation extends to the lateral sinuses or meninges they usually become positive, showing hemolytic streptococci as a rule.

For organisms commonly or occasionally found in blood culture see Microorganisms Found in the Blood.

Sterile technic to avoid contaminants, selection of media that will support growth of the suspected organism, incubation under aerobic or anaerobic conditions or under increased CO_2 tension are of utmost importance.

Contaminants

Of 1,143 blood cultures Fox and Forrester (1940) found diphtheroids as contaminants in 19 per cent and skin cocci in 24 per cent. Of 515 cultures Scott (1951) found 4.7 per cent contaminants consisting chiefly of microaerophilic diphtheroids and coagulase negative *Staphylococcus albus.*

Usual contaminants are coagulase negative *Staphylococcus albus,* diphtheroids, *Bacillus subtilis,* molds, sarcinae, *Proteus* and *E. coli.* When found they should be checked by repeating the culture.

Media and Methods

Many media have been employed. Hormone broth with 0.2 per cent glucose, peptone colloid medium, tryptose-phosphate broth, Kracke's blood culture media, which is designed to fix complement in the patient's blood thus allowing any organism to grow unrestricted, and dextrose (0.2 per cent) infusion broth and agar are satisfactory.

Except for special occasions, dehydrated media prepared by the Difco Laboratories of Detroit and the Baltimore Biological Laboratory (BBL) of Baltimore, Md., are satisfactory and time saving. Composition of each and directions for preparing are given on the container.

A large number of methods have been proposed and the technic employed has considerable influence upon results. Aerobic methods are generally used but unquestionably many falsely negative results occur because of failure to use anaerobic methods. Strictly anaerobic methods should be employed alone or in conjunction with aerobic methods on all suspected cases of puerperal sepsis, with special reference to hemolytic streptococci and *Cl. perfringens,* as well as in all suspected cases of the postoperative septicemias in relation to hemolytic streptococci and organisms of the genus *Bacteroides.*

It is extremely important for successful isolation of organisms from the body fluids of patients receiving sulfonamides that with the exception of thioglycollate-glucose broth, para-aminobenzoic (P.A.B.) acid be added during preparation to all routine culture media in a concentration of 5 mg. per 100 ml. This has been found to inhibit the bacteriostatic action of the sulfonamides and to overcome the difficulty

encountered in isolating the organisms. Similarly, if penicillin has been used, penicillinase must be added to the medium in a final concentration of 1 unit for each 10 ml. One ml. of Bacto-Penase will inactivate 50,000 units or more of penicillin. In culturing blood or other body fluids containing penicillin it is recommended that 1 ml. of Bacto-Penase be added to each 100 ml. or less of culture media. This concentration will inactivate the maximum amount of penicillin that would be present in 10 ml. of blood or other body fluid.

Penicillinase stock solution will keep three months under refrigeration and may be added to large amounts of media to be stored in this manner. Penicillinase, in powdered form, may be purchased from the Schenley Laboratories Inc., 350 Fifth Avenue, New York 1, New York. Bacto-Penase is prepared by the Difco Laboratories of Detroit.

Techniques

The author's laboratory routinely employs 125-ml. flasks containing fluid thioglycollate broth and fluid trypticase soy broth and prefers to add blood directly to the media at the bedside. Each flask contains 50 to 60 ml. of media and no anticoagulants are added. Other workers, however, prefer to use citrate solution (2 per cent in physiological salt solution) for subsequent laboratory culturing. Approximately 5 cc. of blood collected at the bedside are added aseptically to each flask and gentle agitation is used to prevent blood clotting. There should be at least one part blood to 12 parts of culture media because blood is bacteriostatic. Penicillinase or Bacto-Penase is added if the patient has received penicillin within the last 72 hours. Unfortunately, there is no technic available at present for the neutralization of the broad spectrum antibiotics although streptomycin can be effectively inactivated by the use of media containing thioglycollate or other reducing substances. Thioglycollate broth maintains anaerobic conditions without the use of seals or anaerobic apparatus. This medium, although fluid, has the great advantage of supporting the growth of strict and facultative anaerobes as well as the majority of aerobes. It is especially useful in subacute bacterial endocarditis for the cultivation of certain fastidious or microaerophilic streptococci which grow poorly or not at all on primary isolation under aerobic conditions. Thioglycollate broth is recommended for the growth of strictly anaerobic species such as clostridia, *Bacteriodes*, anaerobic streptococci, etc. It should not be stored in the refrigerator because the duration of anaerobiosis is decreased but is best stored at room temperature.

In addition to thioglycollate broth, the author's laboratory routinely

uses trypticase soy broth, because this medium is also capable of supporting the growth of a wide variety of microorganisms including pneumococci, streptococci, meningococcus, *Salmonella spp.*, *Brucella* and members of the genera *Corynebacterium*, *Pasturella*, *Erysipelothrix* and *Hemophilus influenzae*. Trypticase soy broth can also be readily adapted for growing anaerobes such as the pathogenic clostridia by the addition of a small amount of agar (0.1 per cent). All flasks are incubated at 37°C., but an atmosphere of 10 per cent CO_2 is required for the growth of *Brucella* and the meningococcus.

1. In drawing blood for culture at the bedside collect the specimen aseptically by turning off electric fans and close all windows, doors and transoms in the patient's room in order to avoid contamination with air-borne organisms. Whenever possible blood should be withdrawn *before* the administration of an antibiotic or just prior to giving the next dose of drug.

2. Arrange flasks, alcohol lamp, sterile cotton or gauze sponges, tourniquet, etc. as near to patient as convenient.

3. Determine site where the needle is to be inserted. As a rule blood specimens are collected from the median basilic vein. In infants and young children the external jugular is usually most accessible, but the blood should be drawn by an experienced pediatric interne. Rigid aseptic technic is required to guard against skin and air contamination. The area for venipuncture should be palpated *before* and not after cleansing. The skin over the selected vein is thoroughly cleansed with 1:1000 aqueous zephiran. The writer has found this disinfectant satisfactory for skin sterilization. Others prefer to paint the site with tincture of iodine followed by 70 per cent alcohol. Use a tourniquet in order to constrict the venous flow in the arm and distend the vein before inserting the needle. If it becomes necessary to palpate the vein again, the operator's finger tip should be cleansed with zephiran or tincture of iodine.

4. With a 10- or 20-ml. wrapped and sterile (autoclaved) Luer syringe and needle (20 gauge) withdraw 10 or 15 ml. of blood, removing the tourniquet before the needle is taken out of the vein. After drawing the blood remove the needle without touching the syringe tip, flame the mouth of the flask after removing the stopper, and immediately introduce 5 cc. of blood into each of the flasks (thioglycollate and trypticase soy broth). Mix by gentle shaking. In the meantime cover the puncture wound with sterile cotton or gauze and flex the arm in order to produce a blood clot.

5. As a rule, qualitative methods are adequate for routine studies

because bacteria are infrequently seen in direct smears of the blood. On rare occasions, it may be desirable to carry out quantitative determinations of the number of organisms per ml. of circulating blood. If a plate count is desired, blood is collected in a flask containing physiological salt solution with 2% sodium citrate. Mix well to prevent clotting.

6. A request slip recording the *patient's name, age, sex, attending physician's name, suspected diagnosis, antibiotics given recently, date and time the specimen was drawn and signature of the interne or technician* should accompany each specimen to the laboratory.

7. Incubate at 37°C. All flasks are inspected daily and Gram-stained smears are prepared from those showing evidence of growth. Subcultures for identification should be made as soon as there is any evidence of growth on blood agar plates, trypticase soy agar, chocolate agar, Endo's agar or SS agar depending on the organism suspected. It is well to note that in the presence of growth prolonged incubation before subculturing may result in autolysis, especially if pneumococci are isolated. If no growth is present in the broth flasks, prepare stained preparations and inoculate blood agar plates after incubation for 2, 7 and 14 days. It is advisable to incubate for as long as 30 days before discarding the original culture flasks because some bacteria, especially if exposed to antibiotics, grow slowly when first isolated. If growth develops, identify the organisms by further procedures.

Examination of Blood Collected in a B-D Vacutainer Culture Bottle

Of necessity the above may sometimes be collected at a distance from the laboratory by the clinician. This procedure is also especially convenient for the small hospital where requests for blood cultures are relatively infrequent. Use of this method is said to reduce the incidence of contaminated blood culture because a "closed system" is employed and laboratory manipulation is minimized (Conner *et al.*, 1951). The blood culture outfit consists of two units: (1) a partially evacuated bottle containing citrated trypticase soy broth medium, and (2) a venipuncture unit. A wide variety of aerobic and faculative or strictly anaerobic organisms can be cultivated. In addition to the examination of blood and blood clots, a B-D Vacutainer Culture Bottle can also be used for the examination of spinal fluid, pleural fluid, joint fluid and other exudates. This blood culture outfit together with directions for use are available commercially (Becton, Dickinson and Co., Rutherford, N. J.).

Clotted Blood

With a pipette remove the serum aseptically from the clot without centrifugation. Place the clot in the barrel of a 10- or 20-ml. sterile

syringe, and with the plunger force it through the tip into a 100-ml. bottle that contains 50 ml. of trypticase soy broth or thioglycollate medium. From the material left in the specimen container, make a stained preparation and inoculate blood-agar plates. Damon *et al.* (1952) have utilized clotted blood successfully for primary isolation of *Brucella* from patients. Continue the examination according to the procedure described for routine blood cultures.

Autopsy Blood Cultures (Burn)

It is the opinion of many that after death bacteriological studies of the blood and tissues are of little value in the diagnosis and interpretation of disease. However, a systematic study of the relation of bacteria found in the blood and tissues of human cadavers, together with an experimental study of the factors controlling post-mortem invasion, revealed the fact that post-mortem invasion of saprophytic bacteria is not as rapid nor as common as was previously believed. Nevertheless, since post-mortem invasion of the blood and organs may occur it is important to carry out bacteriological examinations as soon as possible after death and always before embalming. A comparative study of blood cultures taken at autopsy and those obtained clinically shows few striking differences, except that the primary infecting organisms in bodies which cannot be cultured soon after death may be accompanied by post-mortem secondary invaders such as *E. coli*, *A. aerogenes*, *Proteus*, *Pseudomonas*, etc. The bacteriological examination of the blood at autopsy can be more significant in the final interpretation of the clinical-pathological correlation, provided rigid bacteriological technic is strictly adhered to during the collection and treatment of the sample of blood at the autopsy table. Most of the unusual findings of the bacterial cultures obtained at post-mortem may be accounted for on the basis of carelessness in the technic at the autopsy table.

Two methods of taking blood at post-mortem have developed which depend upon the time the sample of blood is taken after the death of the individual. In both instances, the blood is obtained from the heart.

Cardiac puncture immediately after death. The area of skin directly overlying the heart is painted with 1:1000 aqueous zephiran or tincture of iodine and alcohol. A sterile large bore needle (18 gauge) attached to a syringe is inserted through the chest wall at about the region of the 4th interspace slightly to the left of the sternum. The heart is penetrated, 5 to 10 ml. of blood withdrawn, and allocated to appropriate media. The media used are those employed for routine blood cultures.

Blood culture at autopsy. A sample of blood is withdrawn from the

pulmonary artery or right auricle after sterilizing the surface of the exposed pulmonary vessel or auricle by searing with a red hot spatula. A sterile large bore needle is inserted through this area and 10 ml. of blood are withdrawn. Occasionally, small fragments of blood clots interfere with the removal of sufficient blood but, by proper manipulation and care, the blood may be obtained. The blood sample is then distributed to the same culture media that are used for blood culture studies. If bacterial endocarditis is suspected remove a small piece of friable vegetation using a sterile forceps and scissors. Wash the vegetation several times with sterile saline and grind with sterile sand or alundum in a mortar containing a few ml. of saline. Make a stained smear of the suspension and inoculate fluid thioglycollate medium. Kolmer *et al.* (1951) state that it is advisable to also inoculate a sodium azide blood agar plate incubated anaerobically in order to inhibit *Proteus* and other Gram negative enteric organisms which are often present as secondary invaders.

General and Special Methods Employed in Blood Cultures

While the general methods given above may be utilized for the isolation of most of the organisms commonly encountered in blood cultures such as streptococci, staphylococci and pneumococci, some of the more fastidious organisms require special procedures or modification of those given above and these are described below.

Special Methods of Culturing

Under Increased Carbon Dioxide Tension

I. **Huddleson's Method.** The stimulating effect of carbon dioxide on *Brucella abortus*, *Neisseria gonorrheae* and *Neisseria meningitidis* has led to the use of various percentages of this gas in culturing these organisms. Furthermore, fastidious organisms such as the meningococcus and *Hemophilus influenzae* can sometimes be recovered more rapidly by daily subculturing to chocolate blood agar plates incubated in an atmosphere of 10 per cent CO_2. Usually an atmosphere containing from 8 to 10 per cent is employed. Several procedures for obtaining this have been described, but two simple methods, Huddleson's and Thompson's are widely used. In Huddleson's method the principle of replacing 10 per cent of the air in a jar containing the cultures with 10 per cent carbon dioxide from a cylinder is utilized. A pressure cooker or Novy jar makes a satisfactory container for the cultures. The volume of the con-

tainer is measured and 10 per cent of the air removed by suction. The amount of carbon dioxide necessary to replace 10 per cent of the air is collected and measured over a column of water. By means of water the gas is forced into the container and the outlets closed. Various modifications of this method are in use (see Fig. 12.1).

Thompson's method utilizes the production of carbon dioxide by the combination of sodium bicarbonate with sulfuric acid. A molar solution of sodium bicarbonate is prepared by dissolving 84 grams in water and making the volume up to one liter. A dilute solution of sulfuric acid is prepared by adding 1 ml. of the concentrated acid to 29 ml. of water. By the combination of these two solutions, part for part, each milliliter of the bicarbonate solution should yield 22.4 ml. of carbon dioxide. Any container which can be sealed is satisfactory. One opening is necessary in the cover. Ordinary museum jars may be used, provided the tops can be fastened securely down and the volume is known. The cultures in tubes or Petri dishes are placed inside the container with a small wide mouth bottle containing the amount of the sodium bicarbonate solution which will yield the necessary per-

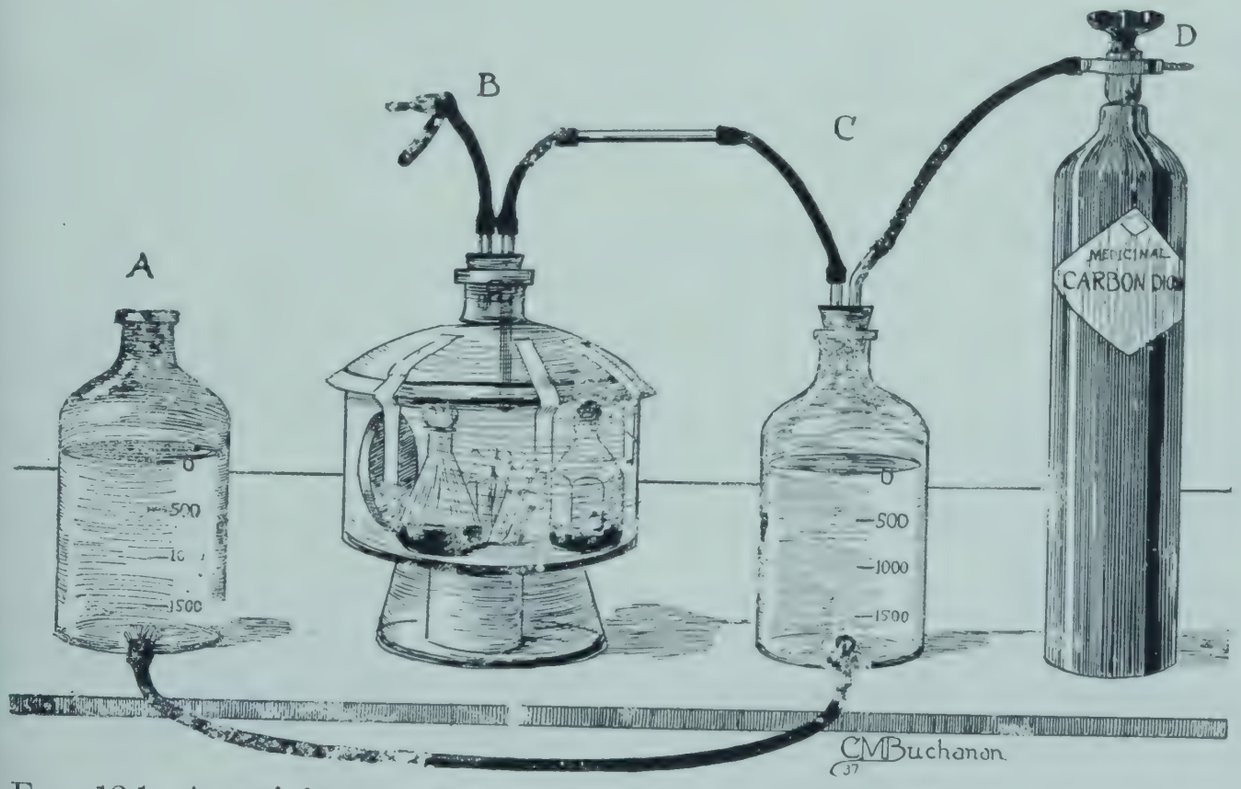


FIG. 12.1. A satisfactory arrangement for cultivating bacteria under partial oxygen tension. Cultures are placed in the large desiccator *B*. Note that the center contains a cardboard carton in which tubes are placed. Around these are placed bottles and flasks on the shelf. To operate, turn the valve on the tank of carbon dioxide forcing the water level in jar *C* down to the mark 500 or more as desired, then release the pinch-cock on the desiccator which permits the measured quantity of gas to replace the oxygen in the jar. The top is sealed with vaseline and adhesive tape.

centage of CO_2. The dilute sulfuric acid solution is pipeted into the carbonate solution through the opening in the cover, then the opening is tightly stoppered as quickly as possible and the jar placed in the incubator.

Shaughnessy (1939) has devised a simplified method for producing increased CO_2 tension in individual culture tubes and flasks. Sulphuric acid with a bicarbonate is used as the source of carbon dioxide. This method may also be applied to anaerobic cultures by using a mixture of pyrogallic acid and sodium hydroxide for oxygen absorption.

II. **Spray Culture Dish.** Completely invert a sterile Spray dish (see Fig. 12.2). Raising the bottom, place 1 ml. of blood in the top, pour enough of the melted and cooled beef infusion agar to fill the top about half full, replace bottom, mix thoroughly by rotary motion, allow to harden, after which invert, thus placing the dish in the upright position, and label. This is to be used for growing organisms requiring an increased CO_2 tension for primary isolation such as *N. gonorrhoeae*, *N. meningitidis* and *Br. abortus*.

Using aseptic technic remove top and pipette into one compartment 1 ml. of a molar solution of sodium bicarbonate (84 gm. to one liter of distilled water) and 1 ml. of a solution of sulphuric acid (1 ml. of concentrated acid added to 29 ml. of distilled water). The sulphuric acid does not need sterilization but the sodium bicarbonate should be boiled or autoclaved before use. The top is then replaced, and sealed to the base of the dish with melted paraffin which is allowed to harden completely. The assembled dish is then tilted slightly and the solutions well mixed. It is then placed in the incubator. The Spray dish may likewise be utilized for anaerobic cultivation using pyrogallic acid and sodium hydroxide in place of the sodium bicarbonate and sulphuric acid (see p. 673).

The dish may be viewed for growth through a lighted electric bulb held on the bottom.

When there is gross evidence of growth in either broth or on the agar culture examine by hanging-drop and Gram-stain. If an organism is found, transplants are made to suitable media and identification is carried out.

III. **The Candle-Jar Method.** Cultures are placed in a large mouth screw top jar. A lighted candle is placed in the jar with inoculated cultures and the top screwed on. When the candle becomes extinguished, oxygen is used leaving sufficient CO_2 to produce growth.

Under Anaerobic Conditions

Anaerobic bacteria may be cultivated in a great variety of media, on slants, plates and in liquids, provided atmospheric oxygen is excluded during incubation. Certain media such as brain broth, whole milk, deep columns of semi-solid agar are more favorable for the growth of anaerobes and often when the oxygen contained in these media is driven off by heating, the organisms will grow without the use of further methods to exclude oxygen.

Thioglycollate medium (Brewer-Modified, B.B.L.) which has already been discussed in this chapter is designed for the "aerobic" cultivation of anaerobes as well as aerobes. Obligate aerobes grow at the top, anaerobes at the bottom of the tube. This medium is highly adaptive for blood cultures and other materials in which the presence of a variety of aerobic, facultative or anaerobic organisms is possible. No more than 25 per cent of the column of fluid medium should be colored pink or blue since this indicates oxidation of the reazurin or methylene blue dye. Thioglycollate medium containing reazurin as the Eh indicator should be used rather than the formula containing methylene blue because the latter dye is toxic for a number of organisms.

Anaerobic thioglycollate agar (B.B.L.) is designed for surface cultivation of anaerobes. Its use in plates with Brewer Petri-dish covers, eliminates, to a large extent, many disadvantages of older techniques (Fig. 12.2).

The incorporated reducing agent, sodium thioglycollate, permits a well poised low Eh potential in the depths of cotton plugged test tubes over long periods.

Because reducing agents are incorporated in the medium anaerobic jars and evaluation-replacement systems are unnecessary. Colonies may be readily observed on the light colored medium and are easily accessible for study.

Techniques for use of the Brewer anaerobic Petri dish cover and anaerobic agar (Baltimore Biological Laboratory, Inc.). In preparation for work with the anaerobic Petri dish covers, they should be placed on

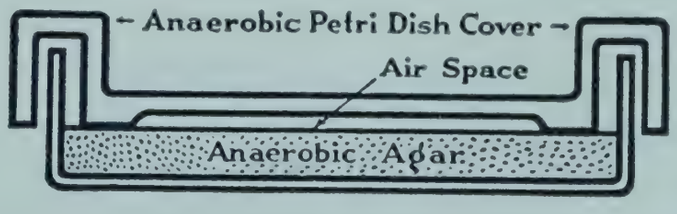


FIG. 12.2. Brewer anaerobic Petri dish cover (B.B.L.)

standard Petri dish bottoms, wrapped in paper, and dry or steam sterilized as preferred.

Melted and cooled (47° to 50°C.) Anaerobic Agar is poured into a regular Petri dish so that the dish is about $\frac{3}{4}$ full. Although not necessary, a porous cover may be used to obtain a dry surface. After the agar has solidified, the dry surface may be streaked in the usual manner, except that the inoculating needle should not be carried to the edge of the plate. Only the area under the air space should be inoculated—about $\frac{1}{2}$ inch of the outer margin of the agar should remain sterile. The regular Petri dish lid is then replaced with a sterile anaerobic Petri dish cover. The cover should not rest on the Petri dish bottom at any point; the inner glass ridge should seal against the uninoculated periphery of the agar. A small amount of air (0.5 to 2.0 mm. deep) is trapped over the central surface of the agar and the oxygen in the air reacts with the reducing agents of the medium, thus creating an "anaerobic" condition.

Alternatively, plates may be made by inoculating sterile cooled and fluid agar before pouring it into Petri-dishes. When the agar has hardened, the regular lids are replaced with anaerobic Petri dish covers.

Examine preparations before incubation. Each plate should be sealed along the inner ridge of the anaerobic Petri dish cover. If the cover touches the agar surface at any point inside the ridge, that is, the central area of air space, the plate should be discarded. The incorporated methylene blue acts as an indicator; in properly prepared plates the reduced center of the medium is decolorized, while the oxygenated edge is blue.

Plates should be inverted and handled by the outer edge of the cover and squeezing of the central part should be carefully avoided. They should be inverted during incubation and may be stacked not more than three high. After growth appears, plates may be opened for selection of colonies and then incubated further if desired.

Some of the methods usually employed for excluding oxygen follow:

For Single Test Tubes or Flasks:

1. The tubes or flasks containing deep columns of liquid or solid medium may be boiled to drive off the oxygen, cooled quickly and inoculated. Then, the medium should be covered with a half inch layer of sterile mineral oil or vaseline to exclude air.
2. For cultures on solid media the oxygen may be absorbed by the alkaline pyrogallate method. The tubes containing the medium must be plugged with a good grade of absorbent cotton, the plug being cut off evenly at the rim of the tube and pushed about $\frac{1}{2}$ inch into the tube. Dry pyrogallic acid is placed in the space above

the plug and 1.5 to 2 cc. of warm 10 per cent sodium hydroxide solution is added by means of a pipette. The tube should be stoppered quickly with a solid rubber stopper and sealed with melted paraffin or parafilm.

For Plates: Isolated colonies on streaked plates of solid agar or blood agar may be obtained by use of the Spray dish. This is particularly useful in isolating anaerobic organisms from fecal material. The Spray dish is a specially designed container made of pyrex glass and consisting of the bottom of a Petri dish over a special dish with a ridge in the bottom and a moat around the top edge. (See Fig. 12.3.) The assembled apparatus is wrapped and sterilized in the hot air oven. The medium is poured into the Petri dish bottom and allowed to harden. Into the special container on one side of the ridge 4 cc. of 40 per cent pyrogallic acid is poured, and on the other side 10 cc. of 20 per cent sodium hydroxide. The medium is inoculated by streaking, the Petri dish replaced and sealed with paraffin or oil clay and the dish tipped to mix pyrogallic acid and sodium hydroxide. The dish is placed in the incubator and when colonies become visible the Petri dish is unsealed and examined.

Anaerobic Jar Methods. Any simple jar with a tight fitting top may be used for anaerobic cultures by employing pyrogallic acid and sodium

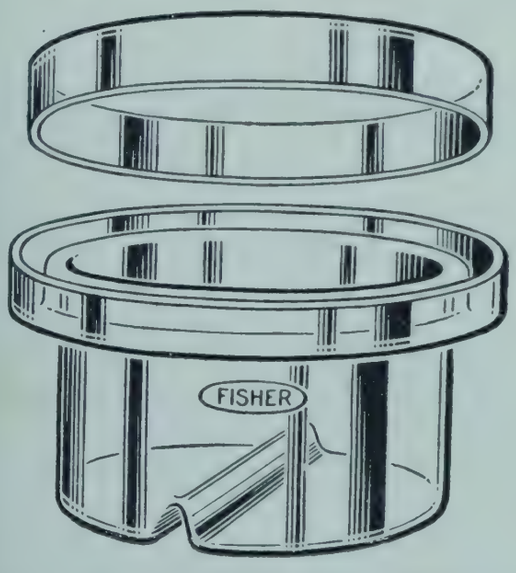


FIG. 12.3. The Spray anaerobic dish. Especially suitable for cultures in Petri dishes. Note the elevated ridge in the bottom. Pyrogallic acid is placed on one side, sodium hydroxide solution on the other, the edge of the Petri dish sealed with vaseline and the chemicals are then mixed by agitation, resulting in exhaustion of the oxygen. Courtesy Fisher Scientific Co.

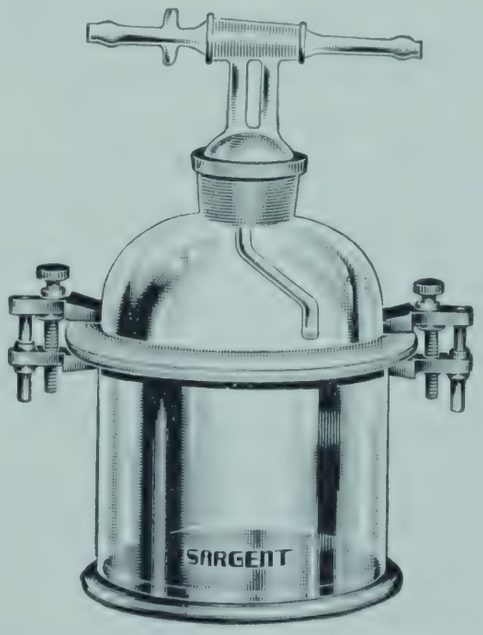


FIG. 12.4. The Novy anaerobic jar for cultures. The air can be exhausted from this type of jar by suction. Courtesy E. H. Sargent Co.



FIG. 12.5. Brewer anaerobic culture jar (Will Corp.)

carbonate. Special jars such as the Novy jar (Fig. 12.4), the McIntosh and Fildes jar, the Brown jar, have been designed to furnish anaerobic conditions for cultures or plates by replacing the oxygen contained in the jar with nitrogen or other gases. These have been widely used but simpler methods such as described by Shaughnessy (1939) are less expensive and just as satisfactory.

The Brewer petri dish cover and the media in dehydrated form may be purchased from the Baltimore Biological Laboratory, Inc., Baltimore, Maryland.

Description: The Brewer anaerobic jar (Fig. 12.5) may be used with either hydrogen or illuminating gas. This is designed to eliminate explosions and operates directly from the lighting circuit that has no needle valves to leak. The current activates a small heating element housed in a metal tube, sealed completely from the inside of the reacting chamber.

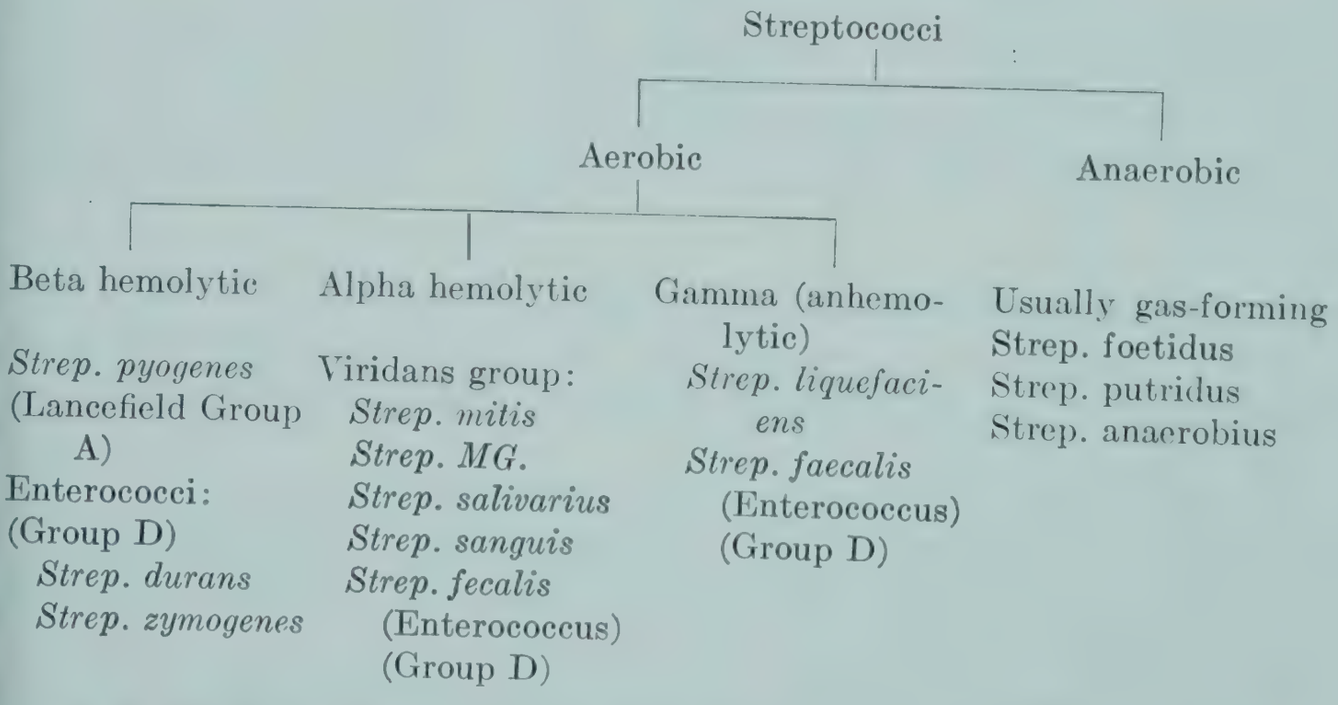
MICROORGANISMS FOUND IN THE BLOOD

Methods of Isolation and Interpretation

1. **Staphylococci.** Isolated by routine method of blood culture. *S. aureus* may be differentiated from albus by its golden yellow color. This may be well brought out by subculturing in infusion agar plates

containing 4 ml. of sterile skim milk to 10 ml. of media or in Löffler's coagulated serum slants. Incubation at room temperature brings out the characteristic pigments. The coagulase test should be used for distinguishing between pathogenic and non-pathogenic strains. Coagulase is an enzyme which causes oxalated blood plasma to clot. Coagulase production is the most important diagnostic property of the staphylococcus because the ability to coagulate plasma (coagulase-positive) is closely correlated with pathogenicity regardless of hemolysis or pigment production. Therefore, all isolated strains should be reported as "coagulase-positive" or "coagulase-negative." *S. aureus* is found more frequently than albus and is usually coagulase-positive. Blood stream invasion by staphylococci occurs in about 50 per cent of the cases involving osteomyelitis of the long bones, also generalized septicemia may sometimes follow an improper or inadequately treated local infection. As previously stated, staphylococci may be recovered from blood in cases of subacute bacterial endocarditis. Isolation is essential to diagnosis and control of therapy.

2. **Streptococci.** Classified according to their action on red blood corpuscles and oxygen requirements as follows:



On a practical working basis the important aerobes are classified according to Brown as:

1. The alpha (viridans) type, producing a greenish discoloration with a narrow zone of hemolysis which under low power microscopically shows many intact red corpuscles in the zone of hemolysis. This zone may increase in size on refrigeration over a 24-hour period.

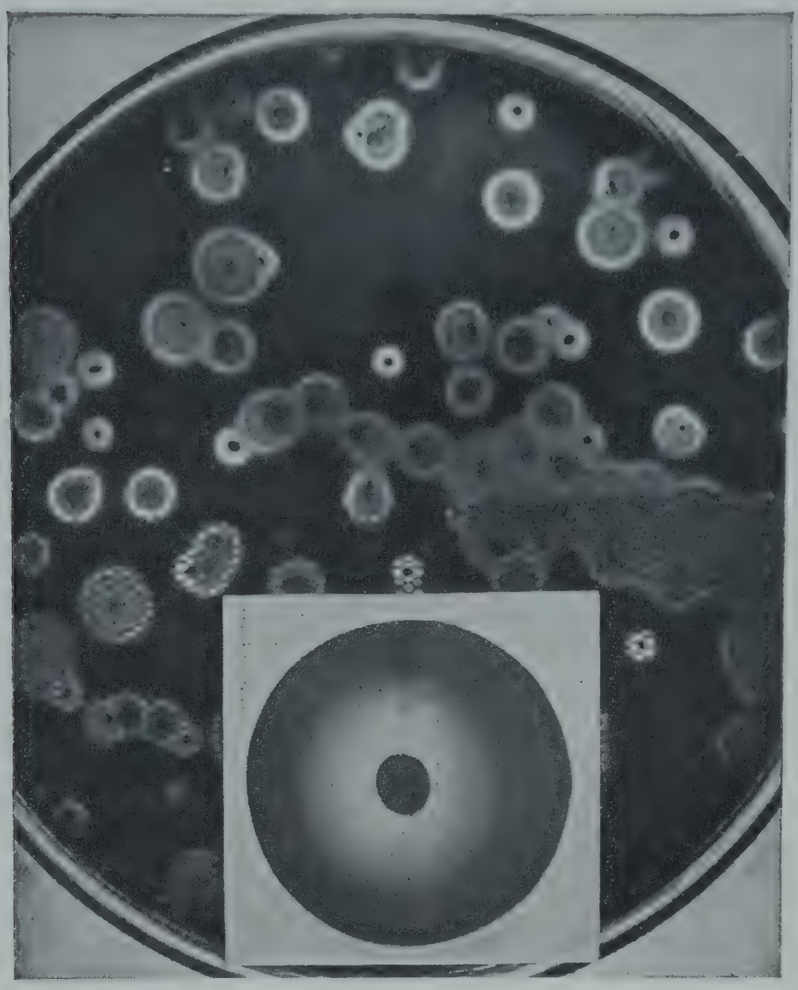


FIG. 12.6. Natural size photograph of blood agar plate showing colonies of beta-type hemolytic streptococci. Each colony is surrounded by a perfectly clear zone in which all the blood corpuscles have been completely destroyed. The insert shows one of the colonies much enlarged. (Courtesy of Dr. J. Howard Brown, Johns Hopkins University.) (Reproduced through permission from Frobisher, *Fundamentals of Bacteriology*, 4th edition, 1949, W. B. Saunders Co., Phila.)

2. The Beta (pyogenes) type which produces a clear zone of hemolysis around the colony which under low power microscopically shows no intact corpuscles with no further extension of this zone on refrigeration (Fig. 12.6).

3. The Gamma (anhemolytic) type which causes no change in the corpuscles. Differentiation into sub-groups may be accomplished by such. Some differential characteristics shown by Frobisher are listed in Table 12.1.

Alpha-hemolytic streptococci (enterococci and the viridans group), other streptococci less frequently, may be found in subacute bacterial endocarditis. Findings are essential to diagnosis, control of therapy and evidence of cure. Members of this group may also cause chronic, suppurative diseases and are frequently found in abscessed teeth, sinuses and tonsils.

The finding of Group A *Beta-hemolytic streptococci* in scarlet fever,

TABLE 12.1

Some Differential Characters of Streptococci

Group Designation	Action on Blood-Agar Plate	Growth at		Survival of 60°C.	Growth in			Representative or Important Species or Varieties	Lancefield Group	Ferments					Acid in Dextrose Broth	Attacks		Human Fibrinolysin	Inulin Fermented	Bile-soluble
		10° C.	45° C.		NaCl 6.5%	*p*H 9.6	0.1% meth. blue.			Lact.	Man.	Sal.	Tre.	Sorb.		Sod. hip.	Gel.			
Beta-type hemolytic streptococci or "*Str. hemolyticus*" (pyogenic group)	*β*	−	−	−	−	−	−	*Str. pyogenes*	A, E, F, G	+	−	+	+	−	little	−	−	+	−	−
								Str. agalactiae	B	+	−	+	+	−	much	+	−	−	−	−
								Str. equi	C	−	−	+	−	−	little	−	−	−	−	−
								"human C"		+	−	±	+	−	mod.	−	−	+	−	−
								"animal C"		+	−	+	−	+	little	−	−	−	−	−
Alpha-type hemolytic streptococci "*Str. viridans*"	*α* (some *γ*)	−	+	− (some +)	−	−	−	*Str. salivarius*		+	−	+			much	−	−	−	−	−
								*Str. thermophilus**		+	−	−			much	−	−	−	−	−
								Diplococcus pneumoniae†		+	−	−			much	−	−	−	+	+
Gamma or indifferent type streptococci or "lactic streptococci"	*γ*	+	−	+	−	−	+	*Str. lactis*‡		+	±	±			much	−	−	−	−	−
								Str. cremoris‡		+	−	±			much	−	−	−	−	−
"Enterococci"	*α* *β* *γ*	+	+	+	+	+	+	*Str. fecalis*		+	+	+	+	+		−	−	−	−	−
								Str. liquefaciens		+	+	+	+	+		−	+	−	−	−
								Str. zymogenes	D§	+	+	+	+	+		−	+	−	−	−

* Distinguished, especially by (*a*) growth at 50° C. and (*b*) failure to ferment maltose, from all other streptococci in this list. Is a great nuisance because resistant to pasteurization.

† Does not grow at 45° C. and always produces alpha type colonies.

‡ These are differentiated by the ability of *Str. lactis* to grow at 40° C. and in media with a salt concentration of 4 per cent or a *p*H of 9.2. *Str. cremoris* cannot grow under these conditions.

§ Beta hemolysis in blood agar.

From Frobisher, Martin, Jr. *Fundamentals of Bacteriology*, W. B. Saunders Co., Phila.

septic sore throat, and other streptococcal infections is an important sign of generalization and danger of metastatic infections. Group A, *Strep. pyogenes* is responsible for the great majority of streptococcal infections of man.

Enterococci. This group of streptococci have certain common characteristics such as wide tolerance to heat and cold, growth in the presence of 6.5 per cent NaCl, bile, pH 9.6, etc. which are usually unfavorable to the other groups of human streptococci. They may be alpha, beta or gamma in type. The enterococci are represented by *Strep. fecalis* which may produce alpha or gamma hemolysis on blood agar plates. Serologically the enterococci all belong to Lancefield Group D regardless of their hemolytic type. Enterococci are frequently isolated from subacute bacterial endocarditis and are usually resistant to penicillin and the sulfonamides. They are normally found in the intestinal tract but may produce urinary tract, abdominal and wound infections.

3. **Pneumococci.** Isolation is the same as for routine blood culture but trypticase soy or dextrose-infusion broth are preferred. Carefully examine for growth 12 to 24 hours following culture, since pneumococci autolyze quickly and may be missed.

At the slightest clouding or evidence of growth make a Gram-stained-smear. In every case, whether or not there is evidence of growth, sub-culture after 18 hours from the blood-broth by means of a streaked blood-agar plate.

Pneumococci are differentiated from beta-streptococci by their lancet-shape, usually occuring in pairs surrounded by definite capsule, bile solubility and fermentation of serum-inulin broth (fermented by most but not all strains of pneumococci). If positive, test is confirmatory, but a negative test does not exclude the possibility of other organisms. The Neufeld or Quellung test (see p. 725), however, is still considered the most rapid and satisfactory method for the identification of an organism as a pneumococcus and may be used for the identification of pneumococci directly from clinical material.

Presence of the pneumococcus in blood findings is a grave prognostic sign, especially late in the disease.

4. **Gonococci** or **Meningococci.** Same as for routine blood culture but use trypticase soy broth and chocolate blood agar. Inoculate media at bedside. Incubate all cultures under 10 per cent CO_2. Examine after 2-3 days' incubation. If no growth and Gram-stained smears of the blood broth are negative, reincubate for an additional 7 days during which examine periodically by

a. Gram-stained smears

b. Streaking one loopful of well shaken blood-broth culture onto freshly prepared chocolate blood agar plates. Incubate under 10 per cent CO_2 for 48 hours.

Due to the debris present and the red background of blood broth it is not an easy matter to detect the *Neisseria* by simple Gram-stain smears. Therefore, subculturing the broth routinely at each examination to freshly prepared chocolate-agar plates with incubation in 10 per cent CO_2 enhances the chances of detection. The *Neisseria* produce an enzyme (oxidase), and an oxidase test is available to detect colonies produced by members of this genus. Once isolated, a *Neisseria* may be identified by cultural, fermentation and serological tests. The meningococci are divided into four serological groups on the basis of agglutination or the Quellung reaction similar to that used for typing pneumococci. The serological groups are I, II, IIa and IV (Branham, 1953).

The presence of gonococcus in blood cultures is important in the diagnosis of gonorrheal endocarditis or arthritis.

Septicemia due to the meningococcus occurring before clinical meningitis may allow early diagnosis. Chronic meningococcemia is rare.

5. **Salmonella** (*S. typhi*, *S. paratyphi A*, *S. paratyphi B*, etc.). Same as for routine blood culture followed by procedures used in isolating these organisms from fecal material.

The presence of Salmonella. In typhoid fever the cultures are usually positive the first week of illness in 85 per cent of the cases. This, therefore, is a means of early diagnosis before agglutinins are present in the blood stream. In paratyphoid fever blood cultures likewise offer an early diagnosis and prognostic information. Bone marrow cultures are often positive.

6. **Shigella.** Procedure same as for the *Salmonella* (blood culture usually negative but may have an important differential value).

7. **Hemophilus influenzae.** Same as for routine blood culture with subsequent subculture (sometimes recovered more rapidly by daily subculture to chocolate blood agar plates with incubation under 10 per cent CO_2). Excellent growth may often be obtained when cross-streaked on one of these media which has similarly been streaked with a culture of *S. aureus* (satellite phenomenon). *H. influenzae* is not often found in the blood stream but it and *H. parainfluenzae* may be recovered in subacute bacterial endocarditis due to either. Isolated small Gram negative bacilli can be rapidly identified as *H. influenzae* by performing a Quellung test with Group A and B typing antiserum. The technic of the test is the same as described for typing pneumococci.

8. **Brucella.** Brucella are Gram negative, short nonmotile, cocco-

bacillary microorganisms. *Br. abortus* and the two closely related species *Br. melitensis* and *Br. suis* cause undulant fever in man. The chief source of infection of *Br. abortus* is unpasteurized cow's milk and infected cattle, *Br. melitensis* infected goats and *Br. suis* infected swine. The three species are very similar but may be differentiated chiefly by cultural methods (Table 12.8). Five ml. of blood are added to a flask containing 60 ml. of trypticase-soy-broth. Incubate at 37°C. for 12 days in an atmosphere of 10 per cent CO_2. Examine at 4-day intervals and subculture 0.5 ml. of the well mixed broth onto a trypticase-soy-agar plate and continue subculturing from the broth every 2 days for 1 month before considering negative.

In *brucellosis* blood cultures are generally positive during the first 2 or 3 weeks but may be positive at any time during the course of the disease. However, they usually become negative as the patient develops agglutinins, complement-fixing antibodies and opsonins. During relapse, cultures may again become positive. In subacute brucellosis, which may follow the acute phase, blood cultures are less frequently positive. In a chronic form of the disease cultures are rarely positive except during acute exacerbations. Many fatal cases of the disease have resulted from infection of the heart valves with this organism giving rise to subacute bacterial endocarditis. Blood or sternal marrow cultures are often the only proof of diagnosis.

9. **Pasteurella tularensis.** Isolation of *Pasteurella tularensis* from the blood is rather unusual. In the opinion of some it is not easy or even possible. Diagnosis of infection from this organism is most frequently made by agglutination tests on the patient's serum.

It should be remembered that this organism is highly infectious, that only those who have obtained immunity through vaccination or having the disease should undertake its isolation. For this reason animal inoculation is not advised.

Inoculate six or seven glucose-cystine blood agar slants and one infusion blood agar slant, which serves as a control, with 0.5 to 1 ml. of the patient's blood (preferably not citrated). Incubate aerobically at 37°C. for a period of from 4 to 10 days, or until growth is observed. Make a Gram-stain and look for extremely small Gram-negative bacilli. If the organism is *P. tularensis*, growth will appear on the cystine media only and the organism is identified by agglutination with specific antiserum. Do not report cultures as negative before three weeks' incubation.

While finding of *Pasteurella tularensis* in blood cultures is rather un-

usual it is advisable that such cultures be made routinely, both in the early stage and at frequent intervals thereafter since a persistence may occur in the typhoid type. Blood cultures may have both a diagnostic and differential value as the typhoid type may be mistaken clinically for typhoid fever or brucellosis.

10. **Bacillus anthracis.** Same as for routine blood culture except that where this organism is suspected in association with possible cutaneous lesions plain agar as well as blood agar plates are used.

Local lesions in man due to *Bacillus anthracis* may often heal spontaneously due to man's relatively high resistance to this organism, but may progress into a septicemia. For this reason one or more blood cultures are always indicated in the presence of large or small lesions. Blood culture may be the only means of diagnosis in the visceral form.

Differential points

B. anthracis	*B. subtilis*
ends square.	ends rounded.
spores near middle.	spores near one pole.
non-motile.	motile (young cultures).
pathogenic for guinea pigs.	non-pathogenic for guinea pig.

11. **Streptobacillus moniliformis.** This organism which is an actinomycete and another, a spirochete, commonly called *Spirillum minus*, may cause rat-bit fever. Both organisms gain entrance into the body of man through the bite of the wild rat which may harbor them in the mouth. *Streptobacillus moniliformis* may cause milk-borne epidemics known as Haverhill Fever, named after Haverhill, Massachusetts where the first outbreak was reported in 1926 by Place and associates. *Streptobacillus* moniliformis may be isolated by repeated blood culture or by sternal marrow culture where blood culture fails. The procedure is similar to that for routine blood culture except that citrated blood is collected and centrifuged. The sedimented erythrocytes are then cultured in infusion broth enriched with 20 per cent ascitic fluid or rabbit serum or in starch dextrose broth containing 20 per cent ascitic fluid (Brown *et al.*, 1942) and incubated at 37°C. in an atmosphere of 10 per cent CO_2. Organisms are found on the surface of red corpuscular sediment as "fluff balls" growth after 24 to 48 hours.

This organism is pleomorphic, showing slender filaments with spherical, oval or fusiform swellings with a tendency to break up into bacillary and coccoid forms. It is usually Gram-negative but young forms may be Gram-positive.

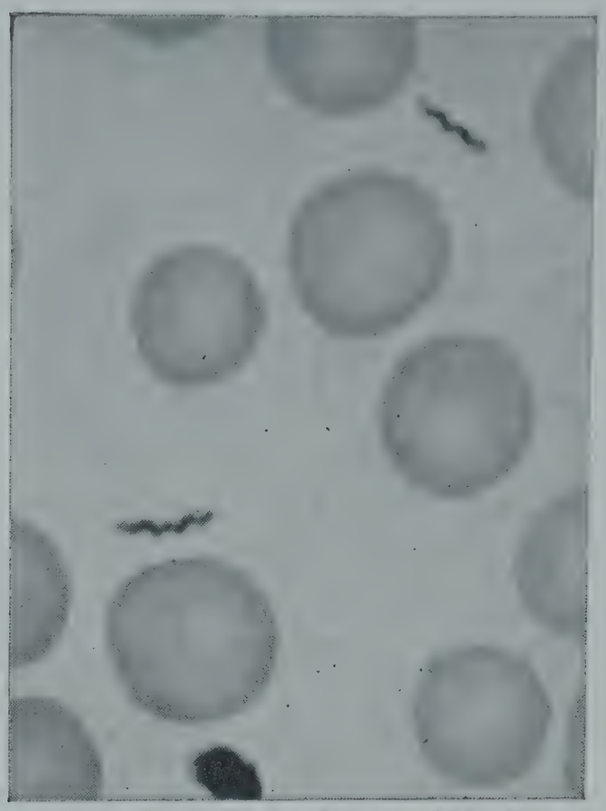


FIG. 12.7. Spirillum minus in blood of inoculated mouse. (Ripley and Van Sant, J. A. M. A., 102,: 1920, 1939).

12. **Spirillum minus.** While the rat is the principal vector of infection caused by this organism cases resembling rat-bite fever have been traced to the bite of the cat, dog, pig, ferret, squirrel and weasel. Ripley and Van Sant reported a case in a medical student who had become infected from a cut during an operation on a dog.

The organism measures 1.5 to 10 micra in length by 0.2 in thickness. It has a rigid body and 1 to 7 flagella at each end. It has $1\frac{1}{2}$ to 9 spirals, each spiral being about 1 micron in length from crest to crest. When viewed in wet preparation the spirillum is seen to have a rapid darting movement frequently changing its direction. It is best seen in fresh blood by Darkfield illumination. When staining with Wright's it takes a dark purple color, flagella are not usually visible (Fig. 12.7).

Diagnosis of the disease caused by this organism is made on a history of rat-bite with symptoms developing after the bite has healed and has later become inflamed. Direct examination of a wet or stained blood smear may reveal the organism. The most accurate method of diagnosis is by injecting the patient's blood or fluid from a local lesion intraperitoneally into white mice, in which the spirilla appear in the blood 3–7 days after examination. This method of diagnosis should be used with caution because white mice have been found naturally infected. The tail blood of mice should therefore be examined for spirilla before inoculation.

13. **Anaerobes.**

a. **Clostridia.** Except for *Cl. perfringens*, clostridia are rarely found in the blood. This may be partly due to the infrequency with which anaerobic methods of blood culture are used. Production of septicemia in man by this organism is not common but invasion is frequent during the agonal period or immediately following death.

Culture is similar to that for other anaerobes and in addition, inoculate 1–2 ml. of patient's citrated blood into a tube of recently heated milk and observe after 24 to 48 hours' incubation in a nitrogen anaerobic jar for a typical, stormy fermentation.

b. **Streptococci.** Anaerobic streptococci should be suspected in cases of septicemias following puerperal fever since finding these organisms is of extreme importance in the diagnosis, prognosis and control of therapy. Outstanding among this group are *S. foetidus*, *S. putridus*, *S. anaerobius*. The urgency of frequent blood culture grown under anaerobic conditions is evident. The method of isolation is similar to that of routine blood culture with the exception that parallel cultures should be grown under aerobic and anaerobic conditions, making possible the isolation of either aerobic or anaerobic streptococci. Use of thioglycollate broth eliminates this duplication as it supports growth of aerobes, anaerobes and microaerophiles. The anaerobic streptococci are chiefly gamma in type and produce gas from carbohydrates as well as a foul odor due to proteolysis.

c. **Bacteroides.** The Gram-negative anaerobic bacilli that do not bear spores have been grouped together under the generic name of *Bacteroides* from the Greek meaning rod-like. Twenty-three species are listed in the 6th edition (1948) of Bergey's Manual of Determinative Bacteriology but only a few have been found to produce pathology in man.

The finding of these organisms is very important since they are capable of producing many infectious processes. In the past their probable presence in septicemias has often been overlooked in many instances because they are easily missed in routine bacterilogic examinations due to the fact that they are anaerobic, grow slowly, often require enrichment with serum, blood or ascitic fluid and glucose in the medium and that growth is often favored in an atmosphere of 10 per cent CO_2.

These organisms are Gram-negative and constitute a large majority of pleomorphic slender, straight, curved and club shaped rods. They are normal inhabitants of the nasopharnyx, gastrointestinal tract, the cervix and vagina. They are not infrequently associated with ulcerated processes of the mucous membranes and may invade the tissues of the

body with the production of abscesses of the liver, lung and other parts of the body, or of the blood stream producing septicemia. They may be present in so called "sterile pus" from surgically drained abscesses and other conditions where bacteria are not found by the usual culture methods.

In their natural habitat these organisms exist as saprophytes. Those that produce pathology may therefore be considered opportunists awaiting favorable conditions for striking man.

Of the many species that have been described *Bacteroides funduliformis* and *Bacteroides fragilis* have been most frequently recovered from the blood in septicemias resulting from various infectious processes. Septicemias caused by them usually arise following throat infections, otitis media, mastoiditis, surgery or infections of the large bowel, genitourinary tract or from war wounds. Septicemias are most frequently due to *Bacteroides funduliformis*. These cases may produce metastatic abscesses that have a high mortality rate. Less frequently septicemias are due to *Bacteroides fragilis*. These run a less fulminating course, with less tendency toward formation of metastatic processes and their prognosis is less grave.

Isolation. May be initially accomplished by growing in thioglycollate glucose broth and agar. Duplicate cultures of each should be prepared; one set grown aerobically, the other under 10 per cent CO_2. Growth is usually evident within 24 hours. If growth is obtained in any culture make Gram-stained smears. Identity may be made as follows:

Bacteroides funduliformis. This organism is highly pleomorphic. The organisms are Gram-negative; slender, straight and curved rods may be found intermingled with filamentous and swollen forms. It is non-motile, non-spore-forming and non-capsulated Figures 12.8 and 12.9. Colonies on blood agar are variable in size from plate to plate and are surrounded by a zone of green hemolysis which may become clear upon prolonged exposure of air. Some strains are non-hemolytic. Broth cultures exhibit diffuse turbidity with production of gas and a foul odor. Colonies on ascitic agar plates are smooth, yellowish-white and convex with an entire edge and from 1 to 1.5 mm. in diameter.

Bacteroides fragilis. This organism is difficult to isolate but will grow on the usual laboratory media. The organisms are small, Gram-negative bacilli, 1 to 2 microns in length and quite regular in size and shape. Curved forms are not infrequently seen. They have a tendency to stain more deeply at the poles. They are non-motile, non-spore-forming and non-capsulated.

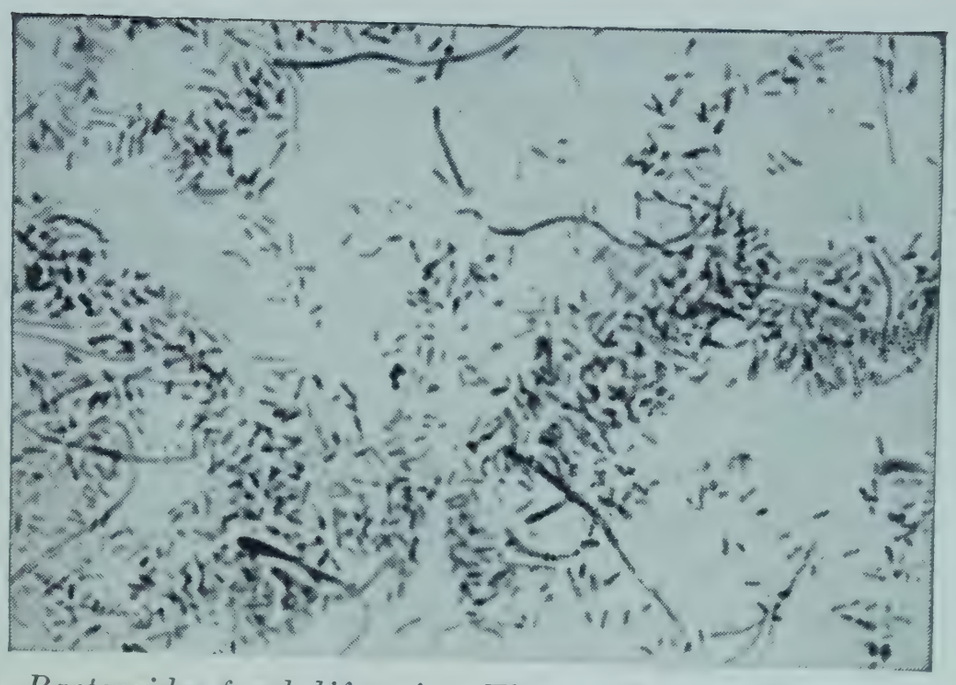


FIG. 12.8. *Bacteroides funduliformis.* The swollen and filamentous forms and poorly staining "ghost cells" are typical of the usual stained smear preparations. ×1000 (Dack).

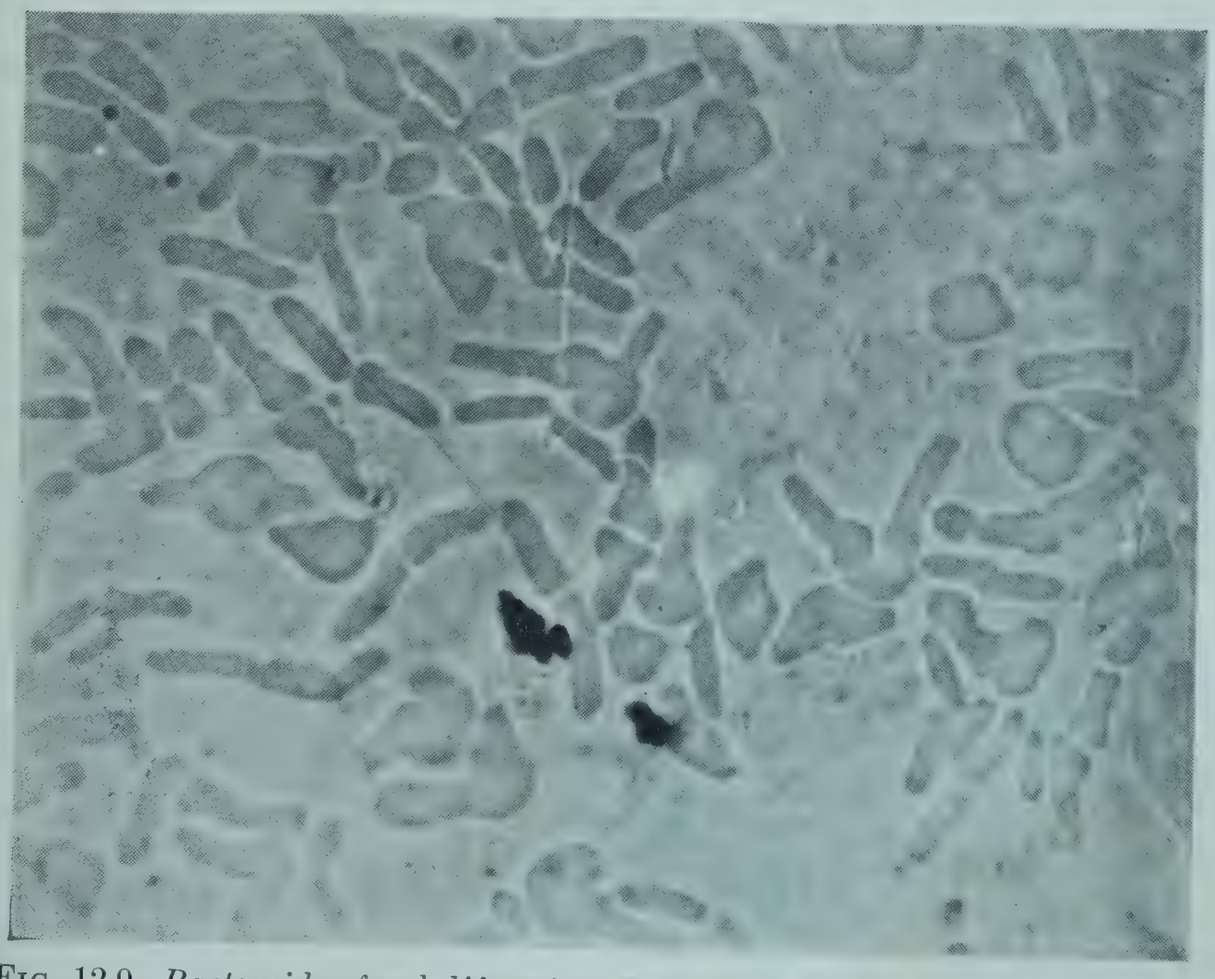


FIG. 12.9. *Bacteroides funduliformis.* Cells from a young thioglycoleate broth culture. The culture is only 9 hours old, yet the cells already show bulbous enlargements and a few large round bodies have formed. There are several pairs of bacilli bespeaking division by fission. One pair in the middle of the photograph shows enlargement of the adjacent ends. The organisms were placed in a thin film of agar and photographed unfixed and unstained with a light microscope. ×1110. (From Smith, Hillier and Mudd, J. Bact., 56: 606, Nov. 1948.)

Colonies on ascitic agar plates are smooth, grayish-white and convex with an entire edge, and measure approximately 1 mm. in diameter. On blood-agar plates, they are non-hemolytic. The organisms are non-pleomorphic, which helps to distinguish them from *Bact. fusiformis*. There is a marked tendency to autolysis with apparent resorption of the colonies and broth cultures are no longer viable after seven to eight days incubation.

Histoplasma capsulatum. This fungus is mentioned here merely for completeness and because it may be cultured from the blood and observed in peripheral blood smears stained by the Giemsa method. A complete description will be found in Chapter 13, Medical Mycology.

Borrelia recurrentis is the etiological agent of relapsing fever. The generic names *Treponema*, *Spirochaeta* and *Spironema* are sometimes used for this group of spirochaetes. Various specific names are often given to strains from different countries, such as *kochii*, *duttonii*, *novyi*, etc., because of differences in vectors or in the clinical course of the disease, but all strains are practically identical in morphology.

These organisms are true spirochaetes since the body is flexible and has no flagella. They are 15–30 micra long and 0.25 micron in thickness. The ends taper to a point. The spirals are relatively coarse, measuring 2–3 micra from crest to crest. They may move rapidly in either direction with a spiral rotation, flexing the body around obstructions, or they may move intermittently from a relaxed irregular position into a straight rigid spiral like a spring which is pulled at both ends. They divide by binary fission, becoming thin at the middle before division takes place. They are moderately refractile and can be found most easily in fresh blood by dark-field illumination, especially if they are not numerous. They stain deep red or purple with Wright's stain in a thin blood film. If the film has dried quickly they are usually caught in abnormal positions without regular spirals, but if the film has dried slowly they retain a straight spiral form. There are often several unstained bands in the body of the spirochaete, or a tapering at the middle if division is about to take place.

Spirochaetes appear in the peripheral blood of patients at about the time of the first rise of fever. They increase usually to enormous numbers in louse-borne relapsing fever, the first attack of which lasts from 5 to 7 days. In tick-borne relapsing fever they are less numerous because the attack lasts only 2 to 4 days. They become agglutinated in the blood stream when the humoral antibodies have developed to a sufficient concentration, and disappear within a few hours with the

critical fall in temperature. They usually cannot be found in the afebrile intervals either by dark-field or in thin blood smears, but may possibly be demonstrated in thick-blood films or by inoculation into susceptible animals. White rats and mice are usually susceptible to the tick-borne strains but less so to the louse-borne strains. In relapses the spirochaetes are usually fewer in number and may not be found at all.

In the United States relapsing fever is endemic only in the Southwestern states and on the Pacific Coast, and is entirely tick-borne. It occurs chiefly in persons who have visited sandy caves or who have slept in huts in the mountains. The important point is to keep the disease in mind. The characteristic fever curve, enlarged spleen and liver, and progressive anemia, following tick-bite or history of possible exposure, are aids in diagnosis.

Leptospira icterohemorrhagiae (Fig. 12.10) is the etiological agent of leptospirosis (Weil's disease, leptospiral jaundice). It is a very delicate spirochaete, measuring 7 to 14 micra in length by 0.25 micra in thickness. The spirals measure 0.5 micra from crest to crest. The organism has the appearance of a string of very fine beads. It is very flexible and the ends are usually recurved forming a C or S shape. The movement is principally rotatory without much foward or backward progress. Multiplication is by transverse fission. The organism will not take the ordinary aniline dyes but can be stained by fuchsin with heat or by Fontana's stain (silver). It is best found by dark field illumination.

Other leptospirae similar morphologically but somewhat different antigenically and producing similar or milder symptoms in man have

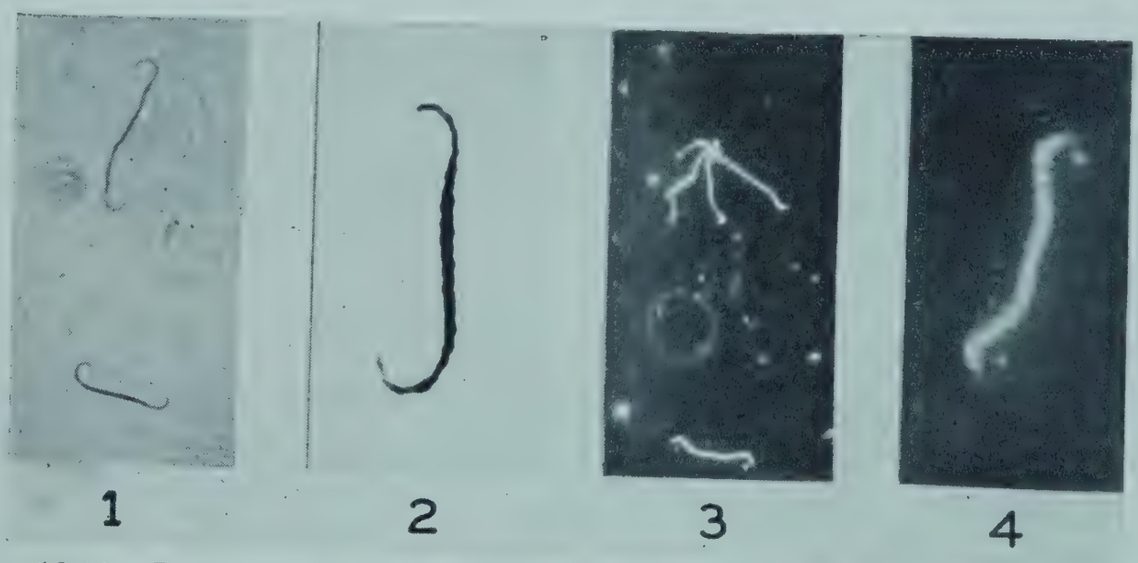


FIG. 12.10. *Leptospira icterohemorrhagiae.* 1 and 2. Osmic vapor fixation, Giemsa stain. 3 and 4. Dark field illumination. 1 and 3, ×1,000. 2 and 4, ×3,000. From Noguchi, H.: J. Exper. Med., 27: 575, 1918.

been found in Japan (*L. hebdomadis*, *L. autumnalis*), and in eastern Europe (*L. grippotyphosa*). In addition a leptospira causing infections in dogs and foxes (*L. canicola*) has been reported in human cases, usually without jaundice. Jeghers (1953) states that in addition to Weil's disease and Canicola fever other leptospiroses found in the United States at present include Swineherd's disease (*L. pomona*), Fort Bragg or Pretibial fever (*L. autumnalis*), Water or Mud fever (*L. grippotyphosa*) and Indonesian Weil's disease (*L. bataviae*).

Rats, dogs and foxes are the reservoir hosts and excrete leptospirae in the urine. Man becomes infected by immersion in water contaminated with rat urine, such as canals, trenches, sewers, fish markets, or by drinking contaminated water. A few cases have occurred from contact with dogs suffering from the disease. Only about one-third of the clinical cases develop jaundice. These cases simulate yellow fever. Leptospirae may be found in some cases in the blood up to the 10th day, after that in the urine sometimes up to the 100th day, or in the liver at autopsy. Guinea pigs and hamsters are very susceptible and can be infected by inoculation of blood or urine, usually developing fatal infection with jaundice. Some strains, however (*L. canicola*, *L. pomona* and *L. grippotyphosa*), as a rule are only slightly pathogenic to the guinea pig (Wolff, 1954). On the other hand, the hamster is more susceptible to inoculations with *L. canicola* than the guinea pig. The patient's serum develops agglutinins of high titer against the specific leptospira. These persist indefinitely after recovery. Recent studies by Beeson *et al.* (1952) indicate the need for leptospiral agglutination tests in all meningeal infections of unknown etiology because the leptospirae of Weil's disease, Canicola fever and Pomona fever are especially likely to cause a meningitis with little or no renal and/or liver involvement and may closely simulate an aseptic meningitis of viral origin (see Chapter 10). Agglutinations will be performed upon request by the Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.

EVALUATION OF BLOOD CULTURES

Blood cultures are often essential in the diagnosis and prognosis of various infectious diseases. The results obtained are sometimes difficult to interpret and correlate with clinical findings. Kotin (1952) states that confusion may be attributed to (1) contamination due to faulty technic in drawing blood or in laboratory procedures, (2) preconceived ideas of bacterial "nonpathogenicity," and (3) variations in blood cul-

ture technics. As already reviewed in the introduction bacteria may be recovered from the blood stream of healthy individuals during physiological states such as normal menstruation and labor. Bacteremia may also be associated with minimal traumatic injuries to the body, tooth extraction, etc. and pathological conditions ranging from minimal local infections to septicemia. Dunham (1933) reported bacteremia in 34 per cent of 115 cultures taken from 49 normal new-born infants. The organisms isolated from the blood of normal individuals are usually strains of low grade or no pathogenicity.

Negative blood cultures are often as much of a problem to the clinician as positive findings. Keefer (1937) states that positive blood cultures are not obtained because the number of bacteria entering the blood stream may be so small and so easily removed by the clearing mechanisms that positive cultures are not obtained. Other bacterial infections in which it is unusual to find positive blood cultures are areas drained by the portal venous system including liver abscess, cholecystitis, appendicitis, peritonitis and pylephlebitis. Dowling states that one of the most puzzling diagnostic problems which confronts the internist is the case which exhibits some clinical features of endocarditis but in which positive blood cultures cannot be obtained. In most cases of this kind the patient is eventually found to have some disease other than endocarditis.

It cannot be overemphasized that preliminary blood culture should be taken before therapy is instituted. The isolation of pathogens such as the beta hemolytic streptococcus, meningococcus, gonococcus, typhoid and other *Salmonella*, *Brucella*, etc. from the blood is of diagnostic significance and usually indicates the presence of a lesion within the tissues. On the other hand, attempts to isolate the etiologic organism from the blood are usually futile in bacillary dysentery, diphtheria, tetanus or tuberculosis.

Bacteriologic Examination of Feces

GENERAL CONSIDERATIONS

The enormous numbers of bacteria usually found in human feces makes a general bacteriologic examination inadvisable. In normal feces, members of the colon group of organisms (*E. coli* and *A. aerogenes*) predominate but *Staphylococci*, *Streptococci* (*Enterococcus*), Gram-positive and Gram-negative anaerobes (*Clostridia* and *Bacteroides*), spiral organisms, yeasts and molds usually are also present. In certain infectious

diseases, such as typhoid and paratyphoid fevers, dysentery, cholera, diarrheas due to *Salmonella* food poisoning, and ulcerative colitis, the pathogenic organisms causing the infection may be isolated, thereby confirming the clinical diagnosis. *Brucella* are rarely isolated in brucellosis, however they have been found to be present over long periods of time in the feces of chronic and recurrent types of the disease. Carriers of the above infections may also be detected by fecal examination. In tuberculosis the causative organism can sometimes be isolated from feces although this is of doubtful significance and does not indicate that the infection is localized in the intestinal tract, since patients with pulmonary tuberculosis often swallow sputum containing the tubercle bacilli. Also nonpathogenic acid fast saprophytic bacilli may be found normally in the feces. The great variety of normal flora present in fecal material, the different characteristics of pathogenic organisms sometimes present, and the wide divergence of diseases which these may cause necessitates the use of special methods and highly selective culture media for a successful bacteriologic study. *Requests for fecal examinations should be accompanied by some information of the clinical symptoms of the patient and should be rather specific in regard to the particular organisms sought.*

COLLECTION OF THE SPECIMENS

Fecal material to be examined bacteriologically is not necessarily collected in a sterile container, but it is essential that it be scrupulously clean. Often the stool is passed directly into a quart jar or a pint sized paper carton, or a procelain jar. However, only a small portion is required for examination. This may be selected and placed into a small container by means of a spatula or wooden applicator. If blood, pus or mucus is present, care should be taken to include portions containing this. The container should be tightly stoppered to avoid the escape of unpleasant odors. It should be delivered to the laboratory as soon as possible.

Using the technic of Hardy *et al.* (1942), specimens may be collected by inserting a dry, sterile swab into the rectum. This is facilitated by incasing the swab in a length of rubber tubing, which when lubricated, easily passes the anal sphincter. Gum rubber tubing (0.5 cm. inside and 0.8 cm. outside diameter) is cut into 12-cm. lengths and one end beveled for about 1 cm. Cotton swabs are prepared with the usual wooden applicator but the cotton must be wound tightly and the end of the applicator well covered. It must readily pass into the rubber tube.

For use, the swab is placed in the tube with the tip slightly short of the beveled opening. The external surface of this end of the tube is lubricated being careful that the jelly does not reach the swab or cover the opening. The tube with swab enclosed is inserted about half its length past the sphincter. The swab is then exposed by withdrawing the tube 2 to 3 cm. The specimen is collected by rotating the swab while sweeping it in a circular motion. The swab is then drawn back into the tube and both are withdrawn. The swab is removed from the tube and used for plating. The tubes are boiled, washed, sterilized and stored. When collecting specimens from patients with watery diarrhea, the tube must be compressed between the fingers to prevent discharge of fecal material.

This method is particularly convenient for culturing large numbers of individuals in a short length of time and for taking cultures on patients in the home and infants and children in pediatrics. Shaughnessy *et al.* (1948), however, have reported that post-cathartic passed specimens are more efficient than rectal swabs as a source of material for the laboratory diagnosis of typhoid and salmonellosis or of the carrier state in these diseases. They also found that the rectal swab is effective in the detection of heavy fecal shedders of *S. typhi* and *Salmonella* and may be of great value in rapid screening of large population groups if combined with subsequent examinations of post-cathartic passed specimens.

In collecting specimens from cases with ulcerative conditions of the colon, sterile cotton swabs on applicators are used to obtain material directly from the lesion through a proctoscope or sigmoidoscope. The swabs tend to dry rapidly, therefore each should be placed in a sterile test tube and cultured as soon as possible.

GENERAL METHODS OF EXAMINATION

If staphylococcal enteritis is suspected, thin smears of the watery feces should be made and stained by Gram's method. In recent years emphasis has been placed on the preponderance of Gram-positive organisms (staphylococci) in severe diarrheas following broad-spectrum antibiotic therapy. The staphylococci are found in large numbers or in pure culture in the liquid feces (see Fig. 12.11), chiefly as a complication of oxytetracycline (Terramycin) or chlortetracycline (Aureomycin) therapy (Jackson *et al.*, 1951; Finland *et al.*, 1953 and Stillwell, 1953).

In a general bacteriologic examination, the fecal specimen is usually diluted by selecting a loopful from different portions and placing each into a tube of sterile saline or broth. Streaks are made from this diluted

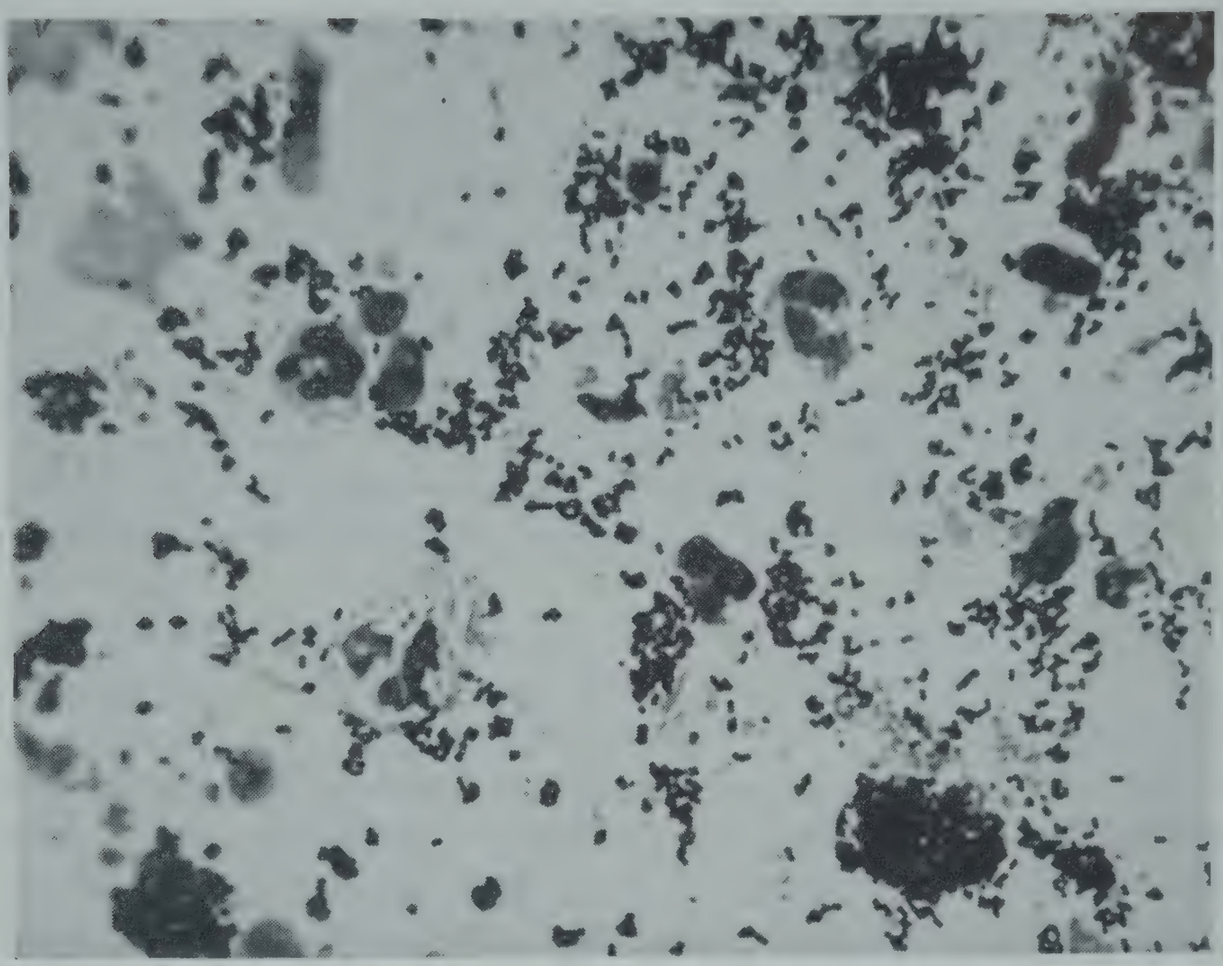


FIG. 12.11 Direct smear of the liquid feces of a pneumonia patient on aureomycin therapy (Gram stain). Note prevalence of staphylococci and polymorphonuclear leukocytes. A pure culture of *Staphylococcus aureus* (coagulase-positive was obtained on culture. (Reproduced through the courtesy of Dr. George G. Jackson of the University of Illinois.)

specimen on plates containing the special selective medias such as Endo's agar, Eosin-Methylene-Blue agar, MacConkey's agar, Wilson-Blair medium, Desoxycholate-Citrate agar or SS agar. Liquid stools may be streaked directly on these plates including a blood agar plate if staphylococcal enteritis is suspected. A description of the colony appearance of the different members of the colon-typhoid-dysentery group is given in Table 12.2 and Plate 12.I.

All cultures should be incubated at 37°C. for 24 to 48 hours. Usually aerobic conditions are employed. Specific methods employed for isolating the usual pathogenic organisms are given in detail.

CULTURE MEDIA

Preparation of many media used in isolating enteric organisms is complex and time consuming. Dehydrated media, because of accuracy of preparation and time saving is most satisfactory for use in isolation and identification of microorganisms from fecal specimens. Since formulae and methods of preparation are given on the package, they are

TABLE 12.2

Appearance of Colonies on Differential Media

Organism	Endo's Agar	Eosin-Methylene Blue Agar	Wilson-Blair $BiSO_3$ Agar	MacConkey's Agar	Desoxycholate Agar	Desoxycholate Citrate Agar	S-S Agar
E. coli	Red colonies, greenish, metallic sheen, media red	Flat, deep purple with metallic sheen	Inhibited for 20 hrs. Large, mucoid, brown greenish, glistening	Brick red with zone ppt. bile	Large, with even red color, milky halo	Inhibited. Red color	Inhibited. Red, pink or colorless. with pink center
A. aerogenes	Red	Light blue, metallic sheen occasionally in depressed center	Raised, mucoid, grayish brown lenticular in shape	Red, mucoid	Large, mucoid. Pink centers, colorless peripheries	Inhibited. Similar to desoxycholate	Inhibited. Large, white or cream colored, opaque
S. typhi	Clear, colorless glistening bluish white	Almost colorless. Translucent	Flat, black with metallic lustre and brownish black zone surrounding	Uncolored, transparent	Clear yellow with raised centers, and irregular thin edges	Large translucent, bluish looking, slightly granular structure	Opaque, transparent, translucent, colorless
S. paratyphi	Clear, colorless	Colorless	Green, variable dark centers	Uncolored, transparent	Yellow, translucent	Yellow, translucent	Same as for *S. typhi*
S. schottmelleri	Clear, colorless	Colorless	Moist black with luster and halo	Uncolored, transparent	Yellow with touch of brown in center	Yellow opaque	Same as for *S. typhi*
S. enteriditis	Clear, colorless	Colorless	Same as *S. schottmuelleri*	Uncolored	Yellow	Yellow opaque	Same as for *S. typhi*
S. paradysenteriae Flexner	Clear, colorless	Small, round translucent	Inhibited	Uncolored	Large, smooth white opaque with even edges	Large, colorless milky opaque, smooth with even edges	Same as for *S. typhi*
S. sonnei	Late fermentation colonies, pink	Colorless	Inhibited	Colorless	Same as Flexner	Inhibited	Same as for *S. typhi*
S. dysenteriae Shiga	Clear, colorless	Colorless	Inhibited	Colorless	Same as Flexner	Inhibited	Same as for *S. typhi*
Alkaligenes fecalis	Colorless	Colorless	Inhibited	Colorless	Large, smooth colonies	Inhibited	Colorless
Proteus vulgaris	Colorless spreading	Colorless	Dark green, clear, dry, no spreading turning black	Colorless	Large, nonspreading touch of brown in center	Inhibited, brown centers	Colorless

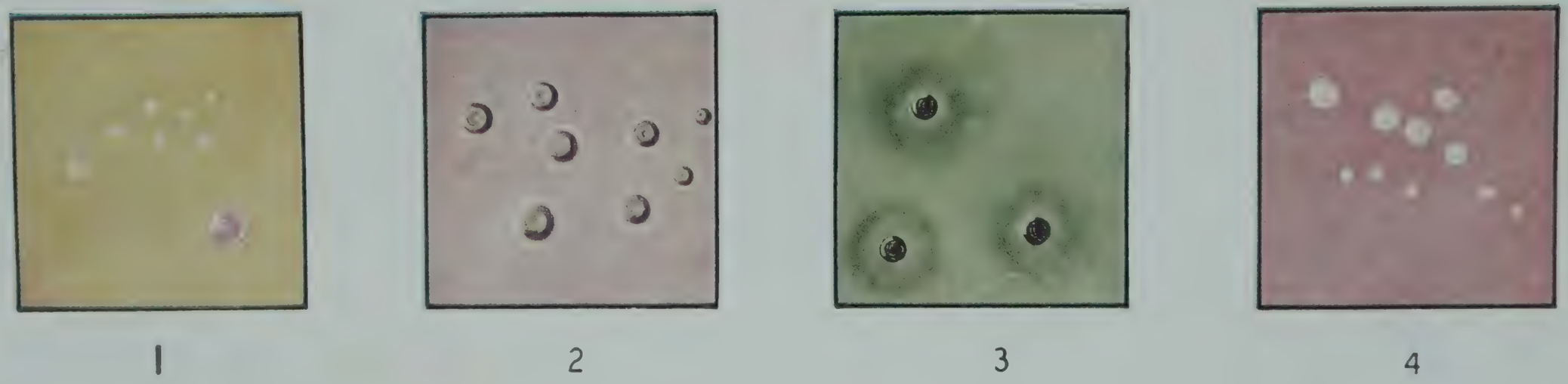




PLATE 12.I. 1. *S. Dysenteriae* (Flexner) on desoxycholate agar 1 colony *E. coli* lower right corner.
2. *E. coli* on eosin methylene blue agar.
3. *S. typhi* on Wilson Blair bismuth sulphite agar.
4. *S. typhi* on Endo's agar.
5. *S. typhi* on desoxycholate citrate agar.
6. *S. typhi* on eosin methylene blue agar.
7. *E. coli* on MacConkey's agar.
8. *E. coli* on Endo's agar.

not repeated here. They may be purchased from the Difco Laboratories, Detroit, Michigan or the Baltimore Biological Laboratory, Inc. (BBL) Baltimore, Maryland.

Free use has been made of the manuals from these laboratories in describing the basis for and use of the various media herein employed.

Culture media employed for the isolation of the *Enteric Bacilli* consists of three principal types:

(A) *Enrichment Media*

Contain substances inhibitory to the growth of certain organisms—*Escherichia*—and promoting that of others—*Salmonella*. Preliminary inoculation of the suspected specimen into liquid enrichment media followed by incubation for 18–24 hours before inoculation on selected plating media, materially increases the percentage of *Salmonella* isolations.

During the first 18–24 hours coliform bacilli are materially reduced, then multiply rapidly. On the other hand *Salmonella* multiply rapidly from the beginning. The *Shigella* are inhibited. *Proteus* and *Pseudomonas aeruginosa* are not.

Two such media commonly used are:

1. Selenite broth
2. Tetrathionate broth base

Selenite Broth. For examining fecal material tubes containing 10–15 ml. of Selenite Broth are inoculated with 1–3 grams of feces or 1–2 ml. of fecal suspension. The specimen is thoroughly mixed in the tubes after which they are incubated at 37°C. for 18 to 24 hours. Following inoculation, a loopful of the enriched culture is streaked on one plate of Bismuth Sulfite Agar and one on a plate of S.S. Agar. These are incubated at 37°C. and examined after 18 to 24 hours. The Bismuth Sulfite plates *should not* be discarded as negative until they have been incubated for 48 hours.

Tetrathionate Broth Base. This is a selective fluid enrichment medium employed in the isolation of *S. typhi* and other members of the *Salmonella* group from feces, urine, water, sewage, and other infected materials. Its use is particularly indicated in the early stages of enteric disorders, in determining the release of patients, and in detecting carriers of typhoid and paratyphoid organisms.

The prepared broth is inoculated by adding 1 to 3 grams of stool, sewage, urine, etc. to 10 ml. of the medium and mixing with a swab, glass rod, or pipette to obtain an even suspension. Many workers find

it advantageous to pass a loosely packed cotton plug down through the inoculated broth to carry the coarser particles of fecal material to the bottom of the tube. The inoculated medium is then incubated for 12 to 24 hours, and streaked upon MacConkey's and Bacto-Bismuth Sulfite agars. The inoculated plates are incubated at 37°C. The MacConkey agar plates are examined after 18 to 24 hours and the Bismuth Sulfite agar plates at 24 and 48 hours for typical typhoid or paratyphoid colonies. Confirm typical colonies on triple sugar iron agar or by other suitable means.

(B) *Differential and Selective Plating Media*

The composition of some media used for identification of these organisms is so adjusted that bacteria give different types of growth upon them. These are called "differential" media. Other media permit the growth of some bacteria while retarding the growth of others. These are spoken of as "selective" media. Such plating media may be used for streaking material direct or from enrichment broth. Developing colonies are subjected to further examination for differentiation of groups and/or species. Those commonly used are:

1. Bismuth-Sulfite agar-Selective.
2. Eosin-Methylene-blue agar, Differential.
3. MacConkey's agar-Differential.
4. S-S (Shigella-Salmonella) agar-Selective.
5. Desoxycholate-Citrate agar-Selective and Differential.
6. Friewer Shaughnessy medium.

Endo's agar was originally developed for the isolation of typhoid bacilli. However, more satisfactory media have been developed for this purpose and Endo's agar has proven of most value in the bacteriological examination of water and milk in confirming the presumptive test for members of the coliform group.

1. **Bismuth Sulfite Agar** (Wilson and Blair medium). Best results are obtained when the medium is dissolved and used immediately. If it is necessary to prepare the medium several days before using, it may be poured into plates and stored in a cold moist atmosphere to prevent drying. After pouring, plates should be kept at room temperature for 1 to 2 hours, then stored in the refrigerator until required. It is advisable to use these plates within four days after preparation. If the medium is to be used immediately following preparation, it should be thoroughly dried by allowing to harden with the covers of the dishes removed. The final reaction of the medium will be pH 7.7±.

On streak plates, colonies of *S. typhi* are black, surrounded by a black or brownish-black zone which may be several times the size of the colony. By reflected light, preferably daylight, this zone exhibits a distinctly characteristic metallic sheen. This sheen may not show where plates are heavily seeded except possibly near the margin of the mass inoculation. In these congested areas, the growth of the typhoid organism is frequently light green. Plates therefore should be inoculated so that discrete colonies may result. *Salmonella paratyphi* (*Paratyphoid A*), *Salmonella typhimurim* (*S. aertrycke*), *Salmonella choleraesuis and Proteus morganii develop upon* this medium yielding flat or only slightly raised green colonies. Generally, members of the *Shigella* group, other than Flexner and Sonne, are inhibited. Those that do develop produce brownish raised colonies with depressed centers and exhibit a crater-like appearance. *E. coli* is usually completely inhibited. Occasionally, a strain will be encountered showing small black, brown or greenish glistening surface colonies. This color is confined entirely to the colony itself and shows no metallic sheen. A few strains of *Aerogenes* may develop, forming raised, mucoid colonies which may exhibit a silvery sheen, appreciably lighter in color than that produced by the typhoid organisms. For appearance of other organisms on this medium, see Table 12.2 and Plate 12.I.

2. **Eosin-Methylene-Blue Agar.** Colonies of *S. typhi*, other *Salmonella*, *S. dysenteriae*, *S. paradysenteriae* (*Flexner*) are translucent and usually grey or colorless on this medium. Colonies of *E. coli* are flat, deep purple with metallic sheen. *A. aerogenes* colonies are light blue, occasionally showing metallic sheen in depressed centers. For appearance of these and other organisms on this medium, see Table 12.2 and Plate 12.I.

3. **MacConkey's Agar.** This medium is used in the detection and isolation of all types of dysentery, typhoid and paratyphoid organisms. Gram-positive bacteria are inhibited.

About 20 ml. of medium should be poured into previously sterilized Petri dishes to form a relatively thick layer. It is quite important that the surface of the plates be quite dry when inoculated. This may be accomplished by allowing the medium to solidify and to stand about 2 hours with the covers of the plates partially removed. Serial inoculation of several plates is recommended to insure suitable distribution of well isolated colonies. After inoculation, plates should be incubated at 37°C. for 16 to 18 hours and should be observed at the end of this period because prolonged incubation may lead to confusion of results.

Coliform bacteria are brick red and may be surrounded by a zone of precipitated bile which is due to the action of the acids, produced by fermentation of lactose, upon the bile salts and the subsequent absorption of neutral red. Typhoid, paratyphoid and dysentery bacilli do not ferment lactose and do not greatly alter the appearance of the medium. Typhoid colonies, in reality giving an alkaline reaction, are uncolored and transparent. When growing in proximity to colon colonies, they have the appearance of clearing the area of precipitated bile. Colonies of dysentery and paratyphoid organisms are similar to those of typhoid. For appearance of other organisms on this medium, see Table 12.2 and Plate 12.I.

4. **S-S Agar.** A restrictive, selective medium recommended for the isolation of members of the *Shigella* and *Salmonella* groups from stools and other materials suspected of containing these organisms.

S-S agar supports well the growth of nearly all the different types of dysentery organisms. The more fastidious Shiga strains do not develop as readily as do strains of Flexner, Sonne, Newcastle, Schmitz and alkalescens. *Shigella*, *Salmonella* and other organisms not fermenting lactose, form opaque, transparent or translucent uncolored colonies, which generally are smooth. The few lactose fermenting organisms which may develop on the medium are readily differentiated due to the formation of a red color in the colony. At times, isolated coliform colonies may not show a definite red color, being pink or nearly colorless with a pink center. Occasionally, an Aerogenes type will develop a rather large white or cream colored opaque characteristic mucoid colony. For appearance of other organisms on this medium, see Table 12.2 and Plate 12.I.

5. **Desoxycholate Citrate Agar**. This medium has many advantages over all others used so far in the isolation and differentiation of intestinal pathogens and for the enumeration of the colon bacillus. For detailed consideration of its uses and values, see Liefson (1935).

The medium may be very heavily inoculated. Plates should be incubated for 24 hours at 37°C. Colonies of colon bacilli, if not inhibited, are red. Colonies of non-lactose fermenters are colorless. Gram-positive bacteria are generally inhibited. A description of the colony appearance of the enteric group of microorganisms is given in Table 12.2 and Plate 12.I.

6. **Friewer Shaughnessy Medium.** This medium is prepared according to the formula of Friewer and Shaughnessy (1944) and is recommended for use as a primary screening medium in the laboratory diagnosis of

enteric infections. It is possible with this single medium to determine motility, lactose fermentation and hydrogen sulfide production. These characteristics are essential criteria in practically all methods of classification of members of the enteric group. This very useful medium is prepared commercially by the Difco Laboratories.

(C) *Differential Media*

Contain carbohydrates and other chemicals into which transplants of suspected colonies from B. are made, the reactions from which aid in the differentiation of genera and/or species. Important among these are:

1. Russell's double sugar agar.
2. Triple sugar iron agar.
3. Various carbohydrate broths.

1. **Russell's Double Sugar Agar.** Russell's double sugar agar is widely used in the identification of Gram-negative organisms belonging to the intestinal group, particularly the colon, typhoid and paratyphoid organisms. It distinguishes the dextrose-acid, dextrose-gas, lactose-acid and lactose-gas forming organisms.

After preparation this medium is used in tubes which are slanted so as to provide a *short slant* and a *deep butt*, the latter providing anaerobic conditions. Inoculation is made by smearing over the surface of the slant and stabbing into the butt. After incubation, the production of acid, both under aerobic and anaerobic conditions, on the slant and in the butt respectively, can be detected by changes in color of the indicator. Gaseous fermentation is indicated by splitting of the agar or formation of bubbles in the butt of the tube. With phenol red as the indicator, production of acid results in a change from the normal pink color of the medium to orange-yellow.

A properly inoculated tube showing, after incubation, a red or cerise slope and a yellow butt, with or without gas formation, indicates fermentation of the dextrose. Some strains of typhoid organisms may require as long as 30 to 40 hours to produce a characteristic alkaline slant. A tube showing a yellow slant and butt with or without gas indicates fermentation of lactose. A tube showing no change indicates that neither dextrose nor lactose has been fermented.

For differential reactions of the enteric organisms on this and other media, see Table 12.3.

Explanation of the reaction on this medium given by Russell is that the bacilli growing aërobically, that is, on the slant, only utilize the car-

TABLE 12.3

Scheme Showing Differentiation of the Enteric Bacilli on E.M.B., Glucose, Lactose, and Russell's Media

Colonies on E.M.B.	Glucose	Russell's media	*For further differentiation of organisms in each group*
1. Deep purple with metallic sheen, or light blue—sheen in depressed center	A. 1. A&G	1. Slant acid, butt acid and gas—colon-aerogenes group	Methyl red Voges Proskauer Indol Simmons citrate Sucrose
2. Colorless	A. 2. A&G	2. Slant, no change, butt acid and gas—paratyphoid-proteus group	Lead acetate Xylose Tartrate Inositol Spreading growth Indol
3. Colorless or translucent	A. 3. A	3. Slant, no change, butt acid only—typhoid-dysentery group*	Motility Rhamnose Mannite
4. Colorless	A. 4. NC B. Lactose 1. A&G 2. NC 3. NC 4. NC	4. Slant, no change, butt no change—alcaligenes group	Gelatin Litmus milk Ferments no carbohydrate

A = acid only; A&G = acid & gas; NC = no change.

* An occasional strain of Newcastle will produce gas on long incubation.

bohydrate when present in amounts over 0.1 per cent, whereas growing anaërobically, stabbed in the butt, they must utilize the carbohydrates for their oxygen supply and, therefore, ferment the trace of glucose present.

2. **Triple Sugar Iron Agar.** This medium was designed by Hajna for the differentiation of the Gram negative enteric bacilli by their ability to ferment dextrose, lactose and sucrose and to produce hydrogen sulfide. This is one of the most useful of the differential tube media in the study of the Gram-negative intestinal microorganisms. It combines the principles of Russell's double sugar agar and Kligler iron agar into one medium which permits differentiation of the Gram-negative rods, both on the basis of their ability to ferment dextrose, lactose or sucrose and or their ability to produce hydrogen sulfide. It differentiates the lactose-splitting organisms from the lactose non-fermenters, distinguishes *Salmonella typhi* from the paratyphoid and dysentery groups, the paratyphoid group from the dysentery group and differentiates *S. paratyphi* from *S. schottmuelleri* and *S. enteritidis*. The inclusion of sucrose per-

mits the separation of proteus organisms such as *Proteus vulgaris* from the salmonellas. The lactose and/or sucrose fermenting organisms-coliforms, paracolons and *Proteus*-develop yellow slants on T. S. I. agar and thus may be differentiated from other species.

Tubes of this medium are inoculated by smearing over the surface of the slant and stabbing into the butt of the tube. The inoculated tubes are observed after incubation at 37°C. for 18 to 36 hours.

Typical reactions of various enteric bacteria on this medium are indicated in Table 12.4.

3. **Various Carbohydrate Broths.** These are prepared as desired by adding the required amount of suitable carbohydrate to stock broth.

THE ENTERIC BACILLI

This constitutes a large group of Gram-negative, non-spore-forming aerobic (optionally anaerobic) bacilli normally or accidentally occurring in the intestinal tract of man and animals in health or in disease. Often referred to as the colon-typhoid-paratyphoid-dysentery group. Impor-

TABLE 12.4

Typical Reactions of Various Enteric Bacteria on Triple Sugar Iron Agar

	Slant	Butt	
		Fermentation	H_2S
Escherichia coli	y.	y.g.	−
Aerobacter aerogenes	y.	y.g.	−
Salmonella typhi	n.c.	y.	+
Salmonella paratyphi (Para A)	n.c.	y.g.	−
Salmonella schottmuelleri (Para B)	n.c.	y.g.	+
Salmonella enteritidis	n.c.	y.g.	+
Salmonella typhimurium (aertrycke)	n.c.	y.g.	+
Salmonella choleraesuis	n.c.	y.g.	+−
Proteus vulgaris	y.	y.g.	+−
Protus morganii	n.c.	y.g.	+−
Shigella shigae	n.c.	y.	−
Shigella ambigua (Schmitz)	n.c.	y.	−
Shigella alkalescens	n.c.	y.	−
Shigella sp. (Newcastle type)	n.c.	y.*	−
Shigella flexneri	n.c.	y.	−
Alkaligenes fecalis	n.c.	n.c.	+−
Paracolon	n.c. or y.	y.g.	+−

* An occasional strain of Newcastle will produce gas on prolonged incubation.
y. = yellow color (acid); g. = gas; n.c. = no change.

tant genera include *Escherichia*, *Aerobacter*, *Salmonella*, *Shigella*, *Paracolon bacilli*, *Proteus*, *Alcaligenes*, and *Pseudomonas*.

They are similar in staining characteristics, grow somewhat similarly on nutrient agar and gelatin. They are differentiated mainly by their action on certain carbohydrates, other biochemical features and agglutination reactions. Comparative differential reactions are shown in Table 12.5.

Coliform Bacilli (Escherichia and Aerobacter)

Escherichia coli and *Aerobacter aerogenes* are considered here primarily for differentiation, by the methods outlined, from pathogens of the enteric group.

TABLE 12.5

*Comparative Differential Reactions of Important Intestinal Bacteria**

	Dextrose	Lactose	Sucrose	Mannitol	Maltose	Xylose	Indole	Gelatin	Litmus milk	Production of H_2S	Russell's medium	
											Butt	Slant
Escherichia coli communis	G	G	–	G	G	±	+	–	ac	–	G	a
Aerobacter aerogenes	G	G	G	G	G	G	–	–	ac	–	G	a
Aerobacter cloacae	G	G	G	G	G	G	±	+	ac	–	G	ak
Salmonella paratyphi (A)	G	–	–	G	G	–	–	–		–	G	k
Salmonella schottmuelleri (*paratyphi* B)	G	–	–	G	G	G	–	–	ak	+	G	k
Salmonella enteriditis	G	–	–	G	G	G	–	–	ak	+	G	ak
Salmonella hirschfeldii (*paratyphi* C)	G	–	–	G	G	G	–	–	ak	+	G	ak
Salmonella typhi	a	–	–	a	a	*a*	–	–	ak	+	a	k
Shigella flexneri	a	–	*a*	a	*a*	*a*	±	–	ak	–	a	k
Shigella shigae	a	–	–	–	–	–	–	–	ak	–	a	k
Shigella sonnei	a	a	a	a	a	–	–	–	ac	–	–	– or k
Alcaligenes fecalis	–	–	–	–	–	–	–	–	k	+	–	k
Proteus vulgaris	G	–	G	–				+	ac?	+	G	k
Proteus morgani	G	–	–	–	*a*	*a*	+	–	k	+	G	k

* G, acid and gas; a, acid only; ac, acid and coagulation; *a*, variable (acid or negative); +, positive (indole production, gelatin liquefaction or H_2S formation); –, no change; ±, variable (acid and gas, or negative); k, alkaline; ak, acid to alkaline. Modified from Burrows, Textbook of Microbiology, 16th ed., 1954.

Escherichia coli

General characteristics. Motile, (young strains and old laboratory strains may show no motility) flagellated, non-spore bearing, sometimes capsulated *aerobic*, facultatively anaerobic, non-chromogenic, non-liquifying, saprophyte, sometimes pathogenic, Gram-negative bacillus, stains by ordinary methods.

Recent evidence presented in the literature indicates that the serotypes of *E. coli* O55 and O111 are closely associated and probably responsible for epidemic and sporadic diarrheal disease of the newly born and infant (Neter *et al.*, 1951). Since *E. coli* serotypes usually do not grow on highly selective media in general use for the isolation of the Salmonella-Shigella group, it is imperative that less inhibitory media such as Endo, MacConkey or eosin methylene blue agar be employed (Ewing *et al.*, 1954). In addition it is recommended that blood agar be included because in a few instances *E. coli* serotypes associated with infantile diarrhea appeared on blood agar plates and did not grow on MacConkey agar. Bray *et al.* (1948) have demonstrated the value of the slide agglutination test for the detection of *E. coli* serological types. This latter finding is of great importance because these organisms are difficult to distinguish culturally from the normal *E. coli* present in feces. The possibility of identifying additional *E. coli* serotypes associated with infantile diarrhea has been discussed by Ewing *et al.* (1954).

Media for Isolation and Growth. Endo's, MacConkeys, Eosin Methylene Blue and Lactose-Litmus agars. Grows profusely on nutrient agar pH 7.4.

TABLE 12.6

*Differential Tests E. coli and A. aerogenes**

	Indol	MR	VP	Citrate
E. coli	+	+	−	−
A. aerogenes	−	−	+	+

* For colonial differences see Table 2.

Aerobacter aerogenes

Similar in most respects to *E. coli*. Primarily a saprophyte found quite regularly in feces of man and animals but in smaller numbers than *E. coli*. Differentiated from *E. coli* by the Methyl Red, Voges-Proskauer tests, growth on sodium citrate medium and indol production.

The Salmonella Group

Salmonella infections in man are of four main types:

1. Typhoid fever due to *S. typhi.*
2. Typhoid-like fevers, called paratyphoid fevers.
3. Acute gastro-enteritis, often referred to as "Salmonella food poisoning" or "Salmonella food infection."
4. A less frequent type of infection is that seen in the involvement of the urinary bladder, pelvis of the kidney, the meninges and the appendix.

Paratyphoid fevers vary from a slight febrile or enteric disorder to a severe fever indistinguishable from typhoid. Salmonella food poisoning differs from enteric fever in its short incubation period, sudden onset, severe gastro-intestinal symptoms, marked prostration and short duration. In spite of the relatively severe symptoms, the fatality is low.

Members of the genus which more commonly affect man are:

1. *S. typhi*, the cause of typhoid fever in man. Characteristic reactions: ferments xylose, dextrose, maltose and mannitol with the production of acid only. *Does not produce gas from any carbohydrates*, H_2S produced in T.S.I. agar.

2. *S. paratyphi*, the cause of paratyphoid A fever in man. Characteristic reactions: does not ferment xylose, rarely able to produce H_2S, fails to utilize citrate and tartrate.

3. *S. schottmuelleri*, the cause of paratyphoid B fever in man. Characteristic reactions: ferments xylose and usually attacks inositol; H_2S formed; citrate + and tartrate usually −.

4. *S. typhimurium* (formerly *S. aertrycke*) a natural pathogen of rodents, especially mice, and many other animals; causes food poisoning in man. Characteristic reactions: very difficult to distinguish from *S. schottmuelleri*, by means of either biochemical or serological reactions, most reliable tests for separating them being: (a) *S. typhimurium* is usually tartrate +; (b) Agglutination reactions with "H" antigens in the specific phase.

5. *S. enteritidis* and its varieties are widely distributed among animals; sometimes the cause of food poisoning in man. Characteristic reactions: ferments xylose, but never attacks inositol; H_2S +, citrate +, and tartrate +. It is differentiated from *S. typhimurium* by serological tests.

6. *S. hirschfeldii*, the cause of a typhoid-like fever in man, sometimes referred to as paratyphoid C. bacillus. Found principally in Eastern Europe and Asia. Characteristic reactions: biochemical reactions similar to those of *S. enteritidis;* serologically, closely related to *S. choleraesuis.*

7. *S. choleraesuis* (*S. suipestifer*) is occasionally the cause of gastroenteritis in man but much more frequently the cause of true enteric fever with invasion of the blood stream. Its natural habitat is the intestinal tract of the hog where it is often found associated as a secondary invader with hog cholera, a virus disease. Characteristic reactions: fails to ferment arabinose, a carbohydrate attacked by other *Salmonella*.

Historical. *Salmonella typhi* was first observed by Eberth 1880 in the spleen and diseased areas of typhoid cadavers. Obtained in pure culture by Gaffky 1884. Found in blood and feces, occasionally in urine of typhoid patients, also in sewage and contaminated surface water, oysters and occasionally ice. Formerly named *Eberthella typhosa* after the discoverer.

General Characteristics. Motile, flagellated, non-spore-forming, aerobic, optionally anaerobic, non-chromogenic, non-liquefying, pathogenic Gram-negative bacillus. Stains by ordinary methods.

Best Medium for Growth and Isolation. Bismuth-sulfite, eosin-methylene-blue, MacConkey's, S-S. For colonial differences see Table 12.2 and Plate 12.I.

Other Salmonella. In 1888, Gärtner described an organism which he obtained from the spleen of a man who had died from eating infected beef and which was also isolated from some of the meat which had been eaten. This organism was given the name of Gartner's Bacillus (*Salmonella enteritidis*).

In 1896, Acharde and Bensuade isolated organisms from urine in a case of apparent enteric fever and from a case of purulent arthritis organisms designated as the paratyphoid bacillus. Schottmueller obtained two bacilli from blood of patients whose symptoms were those of enteric fever. Later, Brion and Kaiser distinguished and named them *Bacillus paratyphosus A* (*Salmonella paratyphi*) and *paratyphosus B* (*Salmonella schottmuelleri*).

S. typhimurium was isolated by De Nobele in 1898 from an epidemic of food poisoning at Aertrycke, Belgium, and *S. choleraesuis* by Salmon and Smith in 1885.

Other species of Salmonella have been described with a total of 153 or more known types.

General Characteristics of the Genus. Gram-negative, non-spore-forming, motile rods, morphologically similar to *Escherichia coli*. They are non-chromogenic, non-liquefying aerobes and facultative anaerobes. Dextrose is fermented, usually with gas production. Lactose and su-

crose are not fermented and indol is not produced. All species are pathogenic for man, animals, or both. Although the chief cause of paratyphoid fever is *S. typhimurium*, *S. paratyphi* B, *S. paratyphi* C and *S. choleraesuis*, most cases of salmonellosis result in gastroenteritis and are largely due to *S. enteritidis*, *S. typhimurium*, *S. montevideo*, *S. oranienburg*, *S. choleraesuis*, *S. panama*, *S. senftenberg*, *and S. anatum*.

The *Salmonella* group are very complex serologically. Each species of *Salmonella* possesses from 1 to 3 distinct antigenic components in the body of the organism (O antigens) and other distinct components in the flagella (H antigens), the latter occurring in many species in two alternate phases, the specific phase and the group phase, each possessing different antigens. The same antigenic components may be found in different species in various combinations. However, most strains of the pathogenic species listed above can be definitely classified on basis of: (a) source of specimen, (b) biochemical reactions, and (c) series of agglutination tests.

These organisms may be differentiated from *Salmonella typhi* by their production of gas in carbohydrate media, while failure to produce hydrogen-sulfide and to ferment xylose may differentiate *Salmonella paratyphi* from the other members of the group, although there may be a variation among different strains. Differentiation among the *Salmonella B* groups must be based on the antigenic formula which becomes quite involved. For clinical bacteriological examination, differentiation of the groups is all that is necessary. For epidemiological purposes, differentiation must be based upon the antigenic formula which involves the determination of the somatic and flagellar antigens. For the latter determinations, it is best to send an unknown strain to Drs. P. R. Edwards in charge of the Salmonella Laboratory, Public Health Service Communicable Disease Center, Atlanta, Ga., or to the State Health Dept. Laboratory.

Biochemical characteristics of the more common members of this group are shown in Table 12.5.

Isolation of Salmonella Typhi and Other Members of Salmonella Group. In cases of typhoid fever the organisms seldom appear in the stools until the middle of the second week of the disease and are more prevalent during the fourth week of convalescence and the carrier state. In paratyphoid fevers the conditions are similar, but in food poisoning infections with *Salmonella* organisms the onset is abrupt and isolations may be made from the feces after the first 24 hours of infection.

Examination of the feces is extremely valuable to the diagnosis of carriers since about 10 per cent of convalescent carriers show the bacilli

for 8 to 10 weeks and 2 to 4 per cent for a year or indefinitely, the latter becoming chronic carriers. The percentage of carriers among females is higher than males. A weekly study of fecal specimens of typhoid carriers for a period of 52 weeks showed that the number of positive stools among female carriers was 15 per cent higher than among males, that both groups were positive 87 per cent of the time and that of 24 weekly specimens of this group typhoid bacilli constituted 49.5 per cent of the bacterial flora.

In Bacillary dysentery examination of stools is of extreme value in the diagnosis of acute, chronic and atypical cases. Often differentiation of Bacillary from Amoebic and Balantidial is only positive by this means. Following recovery from dysentery the carrier state is not as frequent as that seen in typhoid fever. In Bacillary dysentery organisms are present in the earliest stages of the disease. The diarrhea begins as a thin, watery discharge, soon losing its fecal character and finally being composed of nothing but pus, mucus threads and blood. Convalescent carriers are not as frequent as in typhoid, dysentery bacilli usually disappearing from the stools within 2 to 6 weeks following recovery. Chronic carriers are most frequently seen after recovery from Shiga than Flexner infections.

Technic of Stool Examination for Salmonella typhi, Other Salmonella and the Shigella.

1. *The Specimen.* This should be fresh. A delay of 12 hours may diminish positive findings as much as 50 per cent. A delay of even 30 minutes in *Shigella* infections decreases the chance of success and a few hours may render specimens useless. If specimens are to be shipped, they should be sent by special delivery. Addition of glycerine or the use of enrichment media are an advantage in this case. Specimens for mailing may be placed in suitable bottles containing about 10 ml. of a sterile solution of 30 per cent glycerine in 0.85% sodium chloride or one of the enrichment media. Typhoid organisms have been found in stools shipped in glycerine-saline and kept at room temperature 29 days after being received (31 days following the taking of specimen). Whatever is used for collection and shipment, 1–3 gm. of the feces are added to 10 ml. of medium.

2. *Collection.* Small amounts may be collected on wooden tongue depressors and transferred to wide-mouth bottles containing the above material or collected on cotton swabs and transferred in a similar manner. When a stool is to be examined for *Shigella,* select material containing blood stained mucus. All such specimens should be sealed with paraffin.

3. *Examination of Specimen.* If the specimen is received in glycerine-

saline or enrichment medium it may be streaked plated directly. If the suspension of the specimen is too heavy, small amounts may be mixed with physiologic saline solution in order to prepare an even emulsion. Judgment must be the basis of dilution and can best be utilized through experience. Usually the dilution should give an emulsion through which a glass rod or newsprint may just be visible when held behind the test tube containing the emulsion. Approximately 10 ml. of sterile saline and 1 or 2 loopfuls of stool are sufficient.

b. After preparing emulsion, mix well in order to break up clumps and allow to settle.

c. From emulsion of suspended *S. typhi* or other *Salmonella* make streak plate cultures on Bismuth Sulfite, Eosin-Methylene-Blue and Desoxycholate-Citrate agars, taking material with the exception of suspected *Shigella* from the surface without disturbing the sediment.

If the specimen has not been received in glycerine-saline or one of the enrichment media then enrichment media should be inoculated by adding 1 to 3 gm. of specimen to 10 ml. of the medium, mixing with a swab or glass rod to produce an even emulsion. The inoculated medium is then incubated for 12 to 24 hours and streaked upon media indicated in c. above. The use of enrichment media has been shown to increase positive isolation of *S. typhi* by 30 per cent and that of other *Salmonella* from 100 to 700 per cent over direct streaking without enrichment.

d. Inoculated plates are inverted, incubated at 37°C. for 12 to 24 hours and examined for typical colonies. For appearance of typical colonies of *S. typhi* or other *Salmonella* on various media see Table 12.2 and Plate 12.I. Select at least 2 typical colonies of suspended organisms and transfer to Russell's lead acetate, or T.S.I. agar tubes, making surface and stab inoculations by thrusting the needle in the upper part of the slope to the extreme butt of the tube. Incubate at 37°C. for 18 to 36 hours. Examine for acid and gas formation to determine organism suggested by the reactions. See Tables 12.3 and 12.4.

e. Where time is a factor, it is customary in many laboratories to do a slide agglutination test directly from suspicious colonies on plates or from the growth on the tubes of differential media. This is done by picking and emulsifying suspicious growths in saline. A macroscopic slide or a microscopic Widal may be run. These are usually confirmed by the macroscopic test-tube method, setting up a number of serial dilutions. See Immunologic Tests, Chapter 11.

Examination of Feces for Other Salmonella and the Shigella. Similar to that for *S. typhi* except that Eosin-Methylene-Blue and S-S agars

are excellent media for streak-plate cultures when other *Salmonella* or the *Shigella* are suspected.

The Shigella Group (Dysentery Group)

Dysentery may be divided into acute and chronic. Amebae are apparently responsible for most cases of chronic dysentery though bacilli of the colon group also play a part.

In temperate climates acute dysentery is very rarely due to amebae, but usually to the bacillus identified by Shiga or to allied strains, later identified by Kruse, Flexner, Park and others.

Shiga, in 1898, first described and isolated the dysentery bacillus from the feces of patients suffering from the disease in Japan. Later, types were isolated by Flexner, Strong, Schmitz, Andrews and Duval. They are differentiated mainly by their ability to produce indol and to ferment different carbohydrates with the production of acid *but no gas* and by their agglutinability with specific antisera. They are listed below with their differential reactions on carbohydrates and ability to produce indol. Some strains of *Shigella shigae* are inhibited on Endo's, some of *Shigella flexneri* on bismuth-sulfite and some of *Shigella sonnei* on desoxycholate-citrate media. The latter, however, favors growth of Flexner and Newcastle types. For primary isolation, use of tetrathionate enrichment broth followed by direct plating on several media (Eosin-Methylene-Blue, S-S and Desoxycholate-Citrate) are best. This lessens the chance of losing strains which may otherwise be inhibited if only one medium is used.

General Characteristics of the Group. Non-motile, non-flagellated, non-spore-forming, aerobic, non-chromogenic, non-liquefying, pathogenic, Gram-negative bacilli. *None produce gas on carbohydrate media.*

Isolation of Group. The isolation of this group of organisms is essentially the same as for typhoid and paratyphoid organisms. Cultural characteristics may be used as a tentative diagnosis from typical clinical cases. To confirm, agglutination with poly- or monovalent antisera must be resorted to.

Best Medium for Isolation and Growth. Eosin-methylene-blue, S-S and Desoxycholate agars. Following isolation all grow well on nutrient agar pH 7.4.

When *Shigella* are suspected a presumptive diagnosis may usually be made from very fresh fecal material through a microscopic study of the cells. Stained cover-glass preparations and smears stained with Wright's stain are used. Blood is present in varying amounts. Neutro-

TABLE 12.7

Differential Reactions of the Dysentery Bacilli (Acid Only)

Species	Manitol	Lactose	Sucrose	Dulcitol	Sorbitol	Rhamnose	Indol
Shigella shigae	−	−	−	−	−	−	−
Shigella flexneri	+	−	±	−	+	±	±
Shigella ambigua (Schmitz)	−	−	−	−	±	+	+
Shigella alkalescens	+	−	±	+	+	−	+
Shigella sonnei	+	+	+	−	−	+	−
Shigella dispar	+	+	+	±	+	+	+

philes form about 90 per cent of the exudate, many showing nuclear degeneration (ringing) with fat in the cytoplasm. Endothelial macrophages are found containing erythrocytes and leukocytes. They undergo toxic degeneration and form "ghost cells." Plasma cells are abundant early in the disease.

Select flecks of blood-stained muco-purulent material for streaking culture plates.

Swabs from proctoscopic examination are streaked in the usual manner. They may be preserved by dissolving 10 gm. sodium citrate, 10 gm. peptone, 5 gm. sodium desoxycholate and 9 gm. sodium chloride in 1000 ml. of distilled water. Add 3 ml. of a 1 per cent solution of sodium monophosphate and neutralized with N/10 NaOH. Add 0.05 gm. p-aminobenzoic acid. Adjust to pH 8.0, tube and sterilize in the Arnold Sterilizer.

Transfer suspicious colonies of Gram-negative bacilli from plates to agar slants and broth. Incubate 24 hours and examine for motility and by Gram stain. The organisms are non-motile, Gram-negative and occur as rods or coccobacilli and singly as a rule.

For identification inoculate Russell's or T.S.I. agar, peptone water for indol, litmus milk and fermentation tubes of dextrose, mannite, lactose and dulcite for acids (gas is not produced).

Final identification rests upon agglutination with known antiserum. Polyvalent serum may be used or monovalent antiserum for respective types. See Immunologic Tests, Chapter 11.

The Paracolon Bacilli

The paracolon bacteria are usually considered to lie between the coliform bacteria and the Salmonella. They may be defined as aerobic, nonsporulating, Gram negative rods which ferment glucose with forma-

tion of gas but are delayed lactose fermenters (5 to 21 days). Occasional strains fail to ferment lactose.

Evidence continues to accumulate in the literature that certain strains of paracolon bacilli are pathogenic for man. Edwards *et al.* (1947) have reported that the so-called Arizona group of paracolon bacteria have pathogenic properties. Fifty-five types have been described. Recently Murphy *et al.* (1950) have described two outbreaks of gastroenteritis apparently caused by a paracolon of the Arizona group. Stuart *et al.* (1943) have evidence of the pathogenicity of another type of paracolon which they designated as "Biotype 32011" but are now known as the Providence group; eight serotypes of this latter strain have been identified. Members of Biotype 32011 have also been isolated by Barnes (1944) from outbreaks of gastroenteritis affecting naval personnel. Another group of paracolon bacilli have been isolated by Stuart *et al.* (1943) in an epidemic of gastroenteritis in infants. Others have described strains of paracolon bacilli isolated from patients with gastroenteritis with Salmonella or Shigella antigens (Darnall 1948). Paracolon bacteria have also been isolated from urinary tract infections, typhoid-like infections, endocarditis and pneumonia.

The Proteus Group

Proteus vulgaris was isolated in 1885 by Hauser from feces, water, sewage and decomposing materials. It has been found in diarrheal stools of infants and is held by some to be the cause of this condition. It has also been reported as the cause of some outbreaks of acute gastroenteritis following ingestion of certain foods. Its relationship to these conditions however has not been definitely established. Bengston (1910) subjected collected outbreaks of acute food poisoning attributed to *Proteus*, to critical analysis and concluded that relationship of this organism to acute food poisoning is entirely lacking in all instances.

General Characteristics. Highly pleomorphic, actively motile, Gram-negative, aerobic, optionally anaerobic bacillus with peritrichic flagella. Does not form capsules or spores.

Grows luxuriently on ordinary media forming characteristic spreading colonies.

Grows best at 30–37°C., but will grow at 20°C.

On gelatin the colonies are characteristically irregular, hence the name.

Gelatin is rapidly liquefied. *Does not ferment lactose* and usually produces acetylmethylcarbinol (Voges-Proskauer Reaction). Indol is not produced. The fact that certain strains of proteus OX_{19} and OX_2 are

agglutinated by the blood serum of individuals infected with typhus fever (Weil-Felix Reaction) makes it of peculiar scientific interest and diagnostic importance (see Chap. 11).

Proteus morganii was first isolated by Morgan in 1906 from patients with summer diarrhea. Havens *et al.* (1930) isolated it from the feces of 49 patients who had a disease resembling paratyphoid fever. They also isolated it from the blood in 6 cases. The serum of each patient agglutinated the strain of the organism obtained from his own feces, urine or blood in titers from 1:40 to 1:2560. Examination of the feces of 2798 healthy adults showed *Pr. morganii* in only 1.2 per cent. Although members of the genus *Proteus* have been isolated from cases of gastroenteritis their significance as enteric pathogens at present remains unestablished. Cherry *et al.* (1946) have presented circumstantial evidence of the etiologic relationship of *Proteus mirabilis* in an outbreak of gastroenteritis. Most workers, however, are reluctant to attribute any significance to the presence of these organisms in various intestinal infections. For example, Neter *et al.* (1941) feel that the pathogenic significance of Morgan's bacillus in summer diarrhea of children and other intestinal infections is doubtful. On the other hand, others have indicated that *Proteus* is of etiologic significance in infant diarrhea on the basis of increased incidence in stool cultures. In the author's opinion such circumstantial evidence is inadequate to incriminate an etiologic agent because Koch's postulates have not been adequately fulfilled.

General Characteristics. Motile, flagellated, non-spore-forming, *non-liquefying*, non-chromogenic, aerobic, Gram-negative bacillus of doubtful pathogenicity. Stained by ordinary methods. Identified principally by its sugar reactions and agglutination with homologous antiserum.

Best Medium for Growth and Isolation of Proteus vulgaris and Proteus Morganii. Bismuth sulfite, eosin-methylene-blue, MacConkey's and S-S agars. They grow well on nutrient agar pH 7.4.

They may be differentiated by their biochemical reactions: *Proteus vulgaris* ferments saccharose forming acid, and gas, liquefies gelatin, but does not produce indol. *Proteus morganii* does not ferment saccharose or liquify gelatin but does produce indol.

Alcaligenes fecalis

Isolated 1896 by Petruschky from feces.

General Characteristics. A motile, flagellated, non-spore-forming, non-liquefying, non-chromogenic, facultative aerobic, non-pathogenic, Gram-negative bacillus. It does not ferment any sugars but produces marked alkalinity. Forms H_2S.

Best Medium for Growth and Isolation. Eosin-methylene-blue and Endo agars.

Pseudomonas aeruginosa

Isolated in 1882 by Gessard who named it *Bacillus pyocyaneus*. It is a periodic inhabitant of the intestinal tract of man, especially following broad spectrum antibiotic therapy. *Pseudomonas aeruginosa* is known to produce an acute or subacute enteritis with or without constitutional symptoms and diarrhea in both children and adults (Stanley 1947). An epidemic of diarrhea of the newborn with a high mortality has also been attributed to this organism (Hunter *et al.*, 1947). On occasion a typhoid-like fever has been produced. Stanley (1947) has reviewed the incidence and serious prognosis of meningitis, endocarditis, pyelonephritis and other infections due to *Ps. aeruginosa*. It has also been found in abundance in drinking water, which on epidemiological grounds, seemed to implicate it in a dysentery-like epidemic.

There is little doubt of its involvment in certain gastrointestinal disorders, especially following broad spectrum antibiotic therapy. It is probable, however, that its pathogenicity under these conditions is determined by the patient's state of resistance rather than virulence of the organism.

General Characteristics. A small slender motile, flagellated, non-spore forming, non-encapsulated, aerobic (some strains anaerobic) exogenous green fluorescent pigment producing, liquefying, Gram-negative bacillus.

Best Medium for Isolation and Growth. Grows well on nutrient agar pH 7.4. After 24 hours incubation shows characteristically large, soft, smooth, grayish, spreading colonies surrounded by a bright greenish fluorescent pigment which has diffused into the medium. After several days the pigment becomes darker imparting a metallic sheen to the surface growth.

Brucella (Slightly Modified from Borman and West)

Isolation of *Brucella* from the feces is attended with obvious difficulties; success is not to be anticipated. However, there are circumstances when the attempt is advisable. The following technic may be employed.

Mix 1 gm. of feces in 50 ml. of sterile physiological salt solution and shake to obtain a homogeneous suspension. Filter the suspension through four layers of hospital gauze to remove gross particles. Centrifuge at 500 r.p.m. for 3 minutes to throw down particles other than bacteria. Remove the supernatant suspension to another tube, and

TABLE 12.8

Cultural Differences of Brucella Species

	CO_2 Required for Growth	H_2S production	Growth in Presence of	
			Basic fuchsin 1:25,000	Thionine 1:50,000
Br. melitensis	−	±	+	+
Br. abortus	+	+	+	−
Br. suis	−	++	−	+

add sufficient agglutinating serum to make a dilution of 1:100. Shake the tube and place it in a 37°C. water bath for 2 hours. Centrifuge at 500 r.p.m. for 5 minutes and discard the supernatant. Resuspend the precipitate in physiological salt solution, shake and recentrifuge. Discard the supernatant. Repeat this procedure twice. Spread the precipitate over the surface of 2 Eosin-Methylene-Blue agar plates containing a 1:100,000 concentration of crystal violet, and 2 Trypticase Soy Agar plates to which the same concentration of crystal violet had been added. Incubate in 10 per cent CO_2 at 37°C. for 72 hours. Aerobic types of *Brucella* will not be inhibited in 10 per cent CO_2. Transfer suspicious colonies to a second Crystal Violet plate if contaminants are present, or to plain Liver Infusion and Trypticase Soy Agar plates if they are not. Identify the organism by agglutination, by hydrogen sulfide production, and by dye tests. See Table 12.8, Cultural Differences of Brucella Species.

General Characteristics of the Group. Small coccoid or short bacillary forms usually occurring singular or in pairs. In cultures short chains may occur. They are non-sporogenous, non-flagellated, and non-motile. Smooth forms are encapsulated. They are Gram-negative. When stained with aniline dyes there is a tendency to bipolar staining. May be identified primarily by cultural differences. The group by agglutination tests. See Serologic Tests, Chapter 11.

Mycobacterium tuberculosis

A fecal examination is sometimes advisable in cases of intestinal tuberculosis and is made for any purulent or mucous particles in the feces. If these are found, smears are made and stained by the Ziehl Neelson method and a search made for acid-fast organisms. Spores take the acid fast stain and should not be confused with tubercle bacilli. If no purulent material is found in the stool, a heavy suspension of feces in

20 ml. of distilled water is made and 4 per cent sodium hydroxide is added to this. The mixture is incubated in a water bath at 45°C. for five to ten minutes or until it dissolves. At the end of the incubation period, the material is neutralized and centrifuged at high speed for one hour and the sediment then examined for tubercle bacilli. For diagnosis the sediment must be injected into guinea pigs and cultured on suitable media because acid-fast bacillary saprophytes may be found in normal feces.

Bacteriologic Examination of Urine

GENERAL CONSIDERATIONS

In infections which involve the urinary tract, bacteriologic findings in the urine are particularly significant, especially since urine is normally sterile. Because of the frequency of contaminating organisms in specimens of urine sent to the laboratory for routine examinations, and because preservatives often are added which inhibit bacterial growth, bacteriologic examinations are not warranted unless especially requested, and only with specific exceptions should they be made on other than catheterized specimens in the female. On the other hand, urine from the male for routine culture is best collected without catheterization as follows: (1) cleanse the meatus and adjacent structures with 1:1000 aqueous zephiran, (2) instruct the patient to void about one ounce of urine into a clean container without contaminating the glans in order to remove the normal bacterial flora from the anterior urethra (discard this urine) and (3) collect the remaining urine in a sterile container. The organisms most often encountered are: *Escherichia coli*, *Staphylococci*, *Aerobacter aerogenes*, *Streptococci*, *Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Paracolon bacilli*, *Mycobacterium tuberculosis*, and *Neisseria gonorrheae*. Sometimes *Brucella abortus*, and other rare organisms such as *Borrelia recurrentis* in relapsing fever and *Leptospira icterohemorrhagiae* in infectious jaundice may be isolated. Any of these may be causing definite pathologic conditions in the urinary tract, and can come from the kidney or kidney pelvis, the ureters, the bladder or the urethra. Before cultural studies are undertaken 10 or 15 ml. of the specimen should be centrifuged in a sterile centrifuge tube, and smears of the sediment made and stained by Gram's method. In a general bacteriologic examination, a search for tubercle bacilli should be included, and at least one smear made and stained by the Ziehl-Neelsen method. If cultures are to be made in thioglycollate or trypticase soy

broth, the concentrated portion of the centrifuged specimen should be inoculated directly into the medium by means of a sterile pipette. Animal inoculations may also be made from this portion.

Urinary antiseptics should not be administered to the patient for a period of 24 to 48 hours before the collection of the specimen.

COLLECTION OF THE SPECIMEN

All specimens should be collected by means of a sterile catheter, except from the male as described above, in a sterile bottle with a sterile stopper or cotton plug. Care should be taken not to wet the plug in transit from the patient to the laboratory. To avoid external contamination, particular care should be taken in the preparation of the external genitalia before inserting the catheter. If the infection is localized in one kidney, ureteral catheterization is necessary, and specimens from each kidney kept separate.

ISOLATION OF SPECIFIC ORGANISMS

Escherichia coli

Escherichia coli is most commonly found in cases of cystitis, although it is also the most easily acquired contaminant and may be of doubtful significance if the specimen has not been collected with care. These organisms often occur in urine after an infection of typhoid fever. They are easily cultured. If the Gram's stain reveals short, plump, Gram-negative rods, a loopful of the sediment from the centrifuged specimen should be streaked on Endo's medium or eosin methylene blue agar, and the plates incubated for 24 hours at 37.5°C. Abundant growth of large lactose fermenting colonies with a metallic sheen are presumptive evidence that *E. coli* is present. Single colonies should be picked up and grown on slants of Russell's double sugar medium,—or one colony should be emulsified in a small amount of sterile saline or broth and a loopful of this inoculated into the differential sugar media. For the cultural reactions see Bacteriologic Examination of Blood and Feces.

Salmonella typhi

In cases of typhoid fever the organisms have not been found in the urine before the fifteenth day of the disease although they are excreted in the urine in 25 per cent of all cases. Examination of the urine during the disease is of little diagnostic value, but during convalescence and after recovery the organisms may be found for months and sometimes

years, therefore their isolation is important in detecting carriers. Considerable albumin occurs in the urine of convalescents and the organisms appear and disappear with the albuminuria. Obstinate cystitis and suppurative processes in the kidney often follow typhoid.

In detecting the organism Leifson's method of culture is recommended.

Place 10 ml. of urine in a sterile test tube and add 0.23 Gm. of selenite-F enrichment in capsule. Incubate for 18–24 hours, then streak one loopful on bismuth sulfite or desoxycholate citrate agar, and incubate for 24 hours.

Mycobacterium tuberculosis

Tuberculosis of the bladder and kidneys often occurs but the presence of an acid-fast organism in urine is not always indicative of infection as *Mycobacterium smegmatis*, a normal inhabitant of the external genitalia, gives a similar staining reaction. Tuberculosis of the kidneys necessitates ureteral catheterization and the importance of keeping the two specimens separate cannot be overemphasized. If uncatheterized specimens are used a 24-hour specimen is preferable and at least 100 ml. of urine should be centrifuged. The supernatant fluid should be poured off. A smear of the sediment is then stained by the Ziehl-Neelsen method. If this shows no acid-fast organisms, cultures should be made on sensitive media. Inoculation of the sediment into a tuberculin negtive guinea pig in the subcutaneous tissue of the groin will sometimes detect the organism when other methods fail. This also serves to differentiate *Myc. smegmatis* from *Myc. tuberculosis* since the former is nonpathogenic. It should be kept in mind that tubercle bacilli isolated from patients receiving isoniazid may be nonpathogenic for the guinea pig (Middlebrook *et al.*, 1953).

Staphylococci

These organisms often cause cystitis but unless the specimen of urine shows numerous organisms and pus cells when the first smear is made from the sediment, a positive culture of coagulase-negative staphylococci may be misleading as these are frequent nonpathogenic contaminants of the skin. These organisms are easily isolated by transferring 5 ml. of the urine or several loopfuls of the centrifuged sediment to a flask of thioglycollate broth, and incubating for 24 hrs. If staphylococci appear in the culture, the species may be identified by appropriate cultural studies including the coagulase test.

Streptococci

Streptococci in urine usually come from infections in the kidney but may come from the bladder itself. The organisms may be isolated by transferring 5 ml. of urine to a flask of thioglycollate broth, or by streaking a blood agar plate with a loopful of the centrifuged sediment and incubating for 24 hours at 37.5°C. The action on blood agar will indicate whether the species is *Streptococcus alpha* (viridans), *Streptococcus beta* (hemolyticus) or *Streptococcus gamma*. Enterococci are frequently isolated.

Neisseria gonorrheae

The presence of these organisms in the urine is of some significance. They are most often found in voided specimens and have been washed out of the urethra during urination. They may be found by Gram's stain of smears made from urinary sediment and appear as intracellular Gram negative cocci. They are usually accompanied by numerous pus cells.

Proteus vulgaris

Although the primary pathogenicity of this organism is slight, it is often found in cases of cystitis and pyelitis associated with renal calculi, or following obstruction of the urinary tract. It grows best at temperatures of 25°C. and can be cultured on any simple culture media. It is readily identified by its spreading colonies and its rapid liquefaction of gelatin.

INTERPRETATION

Pus and bacteria are significant and can come from any part of the urinary tract. If there is a question of renal or ureteral or other unilateral pathology, a complete cystoscopic examination with catheterization of both ureters and collection of urine direct from each kidney for examination should be made. In evaluating the significance and source of blood, pus, bacteria, and trichomonas in a voided sample of female urine, contamination from vaginal secretions is always possible. Likewise, one should be readily cognizant of the prostate as a source of blood cells, pus, bacteria, and even trichomonas in the voided sample of male urine.

Bacteriological findings are apt to be of little significance on a voided specimen except for finding *M. tuberculosis* or *N. gonorrhea*. When an infectious process is suspected, a fresh catheterized specimen should be

obtained for study except in the male as described above. Fresh urine should also be examined microscopically to ascertain the presence of pus cells as well as the number of bacteria present.

Bacteriologic Examination of Sputum

GENERAL CONSIDERATIONS

Sputum specimens for bacteriologic study are most often received from patients with tuberculosis, pneumonia, bronchiectasis and lung abscess. Since saliva is the normal secretion of the salivary glands and contains the normal mouth flora, care should be taken to obtain sputum free of saliva. As a rule the early morning sputum specimen from a fasting stomach is preferred both for tuberculosis examination and routine culture on a blood agar plate. Some clinicians, however, prefer to collect a 24-hour specimen for tuberculosis examination but this is not suitable for routine culture. The patient should be instructed to rinse the mouth with water before coughing. Sputum should be collected in a sterile container containing *no preservative* and sent to the laboratory at once or stored in the refrigerator. If bronchial infection is suspected, bronchial aspiration is preferred for culture because this technic avoids mouth contamination and is, therefore, more satisfactory for sputum collection.

Bacteria found in the sputum consist of varied types pathogenic and nonpathogenic. A number of species coming from the mouth are normally present and include coagulase-negative staphylococci, alpha and gamma streptococci, nonpathogenic Neisseria, diphtheroids and various treponema (macrodentium, microdentium); *Proteus*, *Pseudomonas*, *Escherichia*, *Aerobacter* and *Serratia* may also be isolated if the patient has received recent oral antibiotic therapy.

Presence of tubercle bacilli (*Mycobacterium tuberculosis*) is evidence of tuberculosis somewhere along the respiratory tract, most probably in the lung. The tubercle bacillus may also cause pneumonia, which may be lobar or bronchial.

Pneumococcal pneumonia is by far the commonest type of pneumonia in ordinary times and comprises the greater bulk of acute infections of the lung.

Pneumococci are responsible for lobar pneumonia in about 96 per cent of the cases, while hemolytic streptococci, Friedlander's bacillus (*Klebsiella pneumoniae*), *Hemophilus influenzae*, coagulase-positive staphylococci and mixed infections are responsible for about 4 per cent. Pneu-

mococci are likewise responsible for about 50 per cent of the bronchopneumonias, streptococci 30 per cent, and 20 per cent are due to miscellaneous organisms. About 25 per cent of the atypical pneumonias are believed to be caused by Beta hemolytic streptococci of the Lancefield Group A and are usually secondary to measles, pertussis, influenza, streptococcus throat infections or scarlet fever. About 10 per cent may be caused by *Staphylococcus aureus* (coagulase-positive) and are usually secondary to similar infections. Both streptococci and staphylococci may be superimposed upon pneumococcal or other pneumonias. Other organisms that may be involved are *H. influenzae* and *H. pertussis;* rarely *Past. pestis* (pneumonic plague), *Past. tularensis*, *B. anthracis* (wool sorters disease), or *Brucella* (especially *melitensis*) may be isolated.

Many of these species may cause abscess of the lung as a sequence to lobar or bronchopneumonia. This is particularly true of pneumonias due to hemolytic streptococci and staphylococci. Kolmer *et al.* (1951) state that undoubtedly the most frequent micro-organisms responsible for lung abscesses are aerobic and anaerobic hemolytic streptococci, *S. viridans*, anhemolytic streptococci, coagulase-positive *Staphylococcus aureus* and *albus*, pneumococci, *H. influenzae*, *Kleb. pneumoniae*, Bacteroides and *Bacterium melaninogenicum*, along with *M. tetragenus* (*Gaffyka tetragena*), *N. catarrhalis* and other diplococci of minor importance, and frequently diphtheroid bacilli, *Borrelia vincentii* or other spirochetes in association with various types of *B. fusiformis.*

Any organism, alone or in combination, causing lung abscesses may likewise be responsible for gangrene of the lung and bronchiectasis, especially anaerobic bacteria, *Borrelia vincentii*, other spirochetes and fusiform bacilli.

Because of their clinical importance and the frequence with which the tubercle bacilli and pneumococci are met with in the examination of sputum, they deserve special consideration in this chapter. Description, recognition of, methods of staining, isolation and identification may be found in the usual text of bacteriology.

DETECTION OF *M. tuberculosis* (TUBERCLE BACILLI)

Preparation and Staining of Smears

Pour the sputum into a Petri dish and place it on a black background. Pick out the opaque particles, particularly bits of caseous or blood-tinged material and transfer to clean *new* slides. For this purpose a pair of tooth picks is very convenient. Several such particles should be ex-

amined. Smears are then made by rubbing the material back and forth on the slide, using a tooth pick or wire loop. Smears about an inch and a half long and about one half inch wide are best. Stain by the Ziehl-Neelsen method.

The property of retaining the carbol fuchsin stain in the presence of acid is spoken of as an "acid fast" characteristic. It is dependent on the presence in the cell of a fatty substance called mycolic acid. This property is not confined to tubercle bacilli but is shared by the leprosy bacillus, smegma bacillus and a number of saprophytic organisms.

When tubercle bacilli are present, they appear as slender, straight or curved rods, staining a solid red color or perhaps showing a beaded appearance. They may appear in groups. At least 10 minutes should be spent in the microscopic examination of each stained slide specimen, and 2 to 4 slides are made of each specimen.

Fluorescopic Method

Richards *et al.* (1941) have developed a simple and efficient method in the examination for acid-fast bacilli, employing the principle of fluorescence microscopy. The bacteria are stained with a fluorescent dye, Auramine O, which when irradiated with ultraviolet light re-radiates as yellow light. When viewed with a fluorescence microscope, the bacteria appear as brilliant yellow organisms against a black or nearly black background.

Advantages. The method is simple, requires no counter staining, consumes about one-third less time for preparing and reading smears, and when bacteria are few and far between, more positive smears will be found than with the usual Ziehl-Neelsen method of staining. Acid-fast bacilli have been found with the fluorescence technic that were not seen in concentrated specimens of sputum and in spinal fluid and pus which were negative with the usual method. However, many workers (Willis, 1944) do not recommend this technic because of the danger of false-positives due to fluorescent particles other than tubercle bacilli.

Interpretation. Acid-fast rods in the sputum usually may be considered to be tubercle bacilli. Their identity must always be confirmed, however, by culture or animal inoculation, especially if there is no correlation with clinical and X-ray findings. In the presence of clinical tuberculosis their absence from sputum may mean that either the concentration of organisms is too low for detection by smear methods or the lesion is not breaking down. It would indicate that the process is in the proliferative rather than the exudative phase. Repeated tests

are often necessary to reveal the organisms. It has been estimated that at least 100,000 bacilli per cubic centimeter of sputum should be present for their detection in the average smear. If a quantitative estimate is required, the average number per field should be reported. To insist that a positive report must be based on the finding of a certain minimum number of bacilli is illogical since only one or more organisms found is adequate for a diagnosis.

GAFFKY'S METHOD FOR RECORDING NUMBER OF ACID-FAST BACILLI (BROWN MODIFICATION)

The following method of recording the number of acid-fast bacilli observed on each slide is widely used.

Gaffky I—only 1 to 4 bacilli in the whole preparation.
Gaffky II—only 1 bacillus on average in many fields.
Gaffky III—only 1 bacillus on average in each field.
Gaffky IV—2 to 3 bacilli on average in each field.
Gaffky V—4 to 6 bacilli on average in each field.
Gaffky VI—7 to 12 bacilli on average in each field.
Gaffky VII—13 to 25 bacilli on average in each field.
Gaffky VIII—about 50 bacilli on average in each field.
Gaffky IX—about 100 bacilli on average in each field.
Gaffky X—enormous masses of bacilli on average in each field.

Concentration Method (Petroff). A quantity of sputum is poured into a sterile tube and measured amount of normal sodium hydroxide solution is added. The volume of NaOH should be about equal to that of the sputum. The tube is then incubated at 37.5°C. for half an hour with occasional shaking. An equal amount of N/1 HCl is then added to neutralize the alkali. Centrifuge the solution at high speed to throw down the bacteria. The sediment can be used for smears, animal inoculation and cultures. It is advisable to smear the sediment in a small drop of 1:10 egg albumin which causes the material to adhere to the slide.

The advantage of this method over some of the other concentration methods is that it can be carried out quickly and does not require washing of the sediment which sometimes results in loss of organisms. The sodium hydroxide destroys the viscosity of the sputum and also kills other bacteria thereby producing a suitable digest for animal inoculation or culture.

Guinea Pig Inoculation. Suitable material can be prepared by concentration as described in the preceding section. It is not advisable to use untreated sputum for inoculation because of the danger of pyogenic

infections and loss of the animal. When fresh, unaltered sputum is used, this is a common result. If possible it is best to inoculate two animals with each specimen. Bovine strains can be differentiated from human strains because they produce generalized and fatal disease in rabbits while human strains cannot do so.

CULTURE METHODS

A positive culture can often be obtained from sputum when the organisms can not be detected by examination of stained smears. Also cultures require a shorter time than is required for pathologic lesions to develop in the guinea pig. Corper estimated that an average of ten to one hundred organisms per cubic centimeter of sputum will produce a positive reaction in guinea pigs or a positive growth on culture. It has been shown repeatedly that cultural methods are capable of detecting smaller numbers of tubercle bacilli than animal inoculation.

The sputum should be treated with a chemical substance which destroys the concomitant bacterial flora but does not affect the tubercle bacillus. Normal sodium hydroxide is an effective agent. Equal portions of the sputum to be tested and the digestant are mixed in a sterile centrifuge tube and incubated at 37°C. for 45 minutes. After incubation, the tube is centrifuged and the supernatant liquid decanted. The concentrated residue is neutralized or washed with sterile normal salt solution, and then cultured on suitable media.

There are many good media for this purpose. Three which give an excellent growth in from one to four weeks are:

1. Lowenstein-Jensen medium
2. Trudeau Committee egg-yolk potato flour medium
3. Petragnani's medium

The concentrated sediment should be spread heavily over the surface of several tubes of medium, using a sterile capillary pipette and with a serpentine motion. If the media is in screw cap tubes, these are firmly applied; if in cotton plugged tubes, they must be sealed with paraffin.

TYPING OF PNEUMOCOCCI

The successful use of various drugs, such as sulfadiazine and penicillin, has largely replaced that of specific antisera in the therapy of pneumococcal pneumonia. Where typing in the past was the rule in most clinical laboratories, it has now become the exception or is not done at all. In spite of this, where possible and practical, routine typing of sputum is justifiable, not only through its academic interest but in

those cases where antiserum alone, or in combination with other therapeutic agents, may be indicated. The individual may have developed an idiosyncrasy to the therapeutic agent or some complication such as nephritis or a dermatitis, indicating the use of antipneumococcic serum.

Typing is generally done by the Neufeld method because it is simple, accurate, quick and inexpensive. It may be done directly on sputum, any fluid or culture containing pneumococci. When pneumococci are not present in sufficient numbers for direct typing, animal inoculation may be necessary. Animal inoculation is likewise useful in corroborating the type found in the sputum or to identify types from blood cultures, spinal fluid, pleural exudate, pus, etc. Because of the present infrequency with which typing is resorted to, only the Neufeld and the Mouse methods are herein considered.

In addition to typing sputum, a blood culture is useful in confirming or refuting the diagnosis of bacteremia, as well as offering a prognostic sign. Also, types of pneumococci identified in the sputum may not be the same as that responsible for the pneumonia and bacteremia.

Collection and Examination of Sputum Preliminary to Typing. The sputum is collected in a sterile container such as a Petri dish, avoiding salivary contamination as much as possible. It should be typed immediately or within an hour or two, as older sputum sometimes fails to give the typical reaction. The administration of sulfadiazine does not interfere with pneumoococcus typing.

Sputum from Infants and Children. It is usually difficult to obtain sputum from children and some individuals. The nasopharyngeal technic of Alexander *et al.* (1941) is recommended for culturing and typing of pneumococci and *H. influenzae* from the nasopharyngeal mucus of children. There is general agreement that nasopharyngeal mucus is superior for typing and culturing than material obtained on pharyngeal swabs. This technic is also recommended for culturing beta hemolytic streptococci and *Staph. aureus*. The technic consists of inserting a stiff copper wire applicator ($5\frac{1}{2}$-inch long and 1 mm. in diameter) with a small cotton swab passed back through the nose to the pharyngeal wall. It is important to allow time for the swab to become moistened after introduction into the nares, before the posterior wall is encountered. On withdrawal the swab is immediately placed in 0.2 cc. of pneumococcus broth and sent to the laboratory for typing by the Neufeld or Quellung reaction (Fig. 12.12) and inoculation of trypticase soy broth and a blood agar plate.

The following stains should be made from selected portions of the sputum.



FIG. 12.12. Showing the capsular swelling of pneumococci in the Neufeld reaction. Left: No change in the capsule. Right: Swelling of the capsule. After Sabin, J. A. M. A. 100: 1584, 1933.

1. Gram's stain.
2. Capsule stain (Hiss or other type preferred).
3. Acid-fast stain.

In the Gram stain is noted the presence of lancet shaped Gram-positive diplococci, the nature of other organisms and cellular elements.

In the capsule stain, the pneumococci will be stained a dark, intense blue while the capsule will appear as a faint blue halo around the organism. Occasionally, when a worker is in doubt as to the degree of capsulation, this stain will bring out the detailed capsular structure. However, it does not clearly stain the capsule in all strains of pneumococci. Its value can be recognized when one considers that any evidence whatever of capsulation is sufficient to place the organism in the pneumococcus group.

In the acid-fast stain, one naturally looks for *M. tuberculosis*. Occasionally, the latter are unexpectedly found in bundles, inasmuch as a patient may be suffering from tuberculous pneumonia or an individual may have a pneumococcal pneumonia superimposed. This preparation, likewise, gives considerable information as to the character of the sputum and usually reveals pneumococci if present.

The Neufeld (Quellung) Technic

In 1902, Neufeld observed that when pneumococci are mixed with specific immune serum there occurs, in addition to agglutination, a swelling ("Quellung") of the capsules. This has been a useful method for the rapid typing of pneumococci. To carry it out successfully, it is essential to use *rabbit* antipneumococcus sera, as the usual immune sera for horses are not effective. Sputum and exudates containing pneumococci may be used for the test.

If Gram stain does not show typical Gram positive lancet-shaped diplococci, repeat the preparation. If 2 to 3 per cent or more of the organisms are characteristic of pneumococci, proceed with typing, which is done as follows:

1. Place a small fleck of blood-tinged or the rusty portion of sputum on a glass slide, using a platinum or a nichrome loop.
2. To the particle of sputum, add 4 to 5 times as much undiluted antipneumococcus rabbit serum (Type I for one preparation; Type II for another, and so on).
3. Add a loopful of Löffler's alkaline methylene blue solution to the sputum-serum mixture.

4. Place a coverglass over the preparation and seal edges with Vaseline or other preparation.

5. Examine preparation with the oil immersion lens and an artificial source of light (preferably a blue light). The reaction should be complete in about 2 to 5 minutes. When the test is positive, the blue pneumococci are seen to be surrounded by wide refractile zones which do not take the stain and have a ground-glass appearance.

Cooper and Walter state that type determination by the Neufeld reaction seems to be the most rapid and reliable method which has been devised. It has the advantage that it can be applied wherever suitable microscopic means are available without other, more cumbersome laboratory equipment. It has the further advantage that the approximate number of pneumococci present can be estimated, which is of some diagnostic and prognostic value. The identification of the organisms actually present in the sputum is more reliable than any method in which mice are employed, because in mice, there is always danger that certain mouth pneumococci, which are more virulent for the mouse, may overgrow the infecting type of the case. Typing sera (rabbit) may be purchased in vials or in capillary tubes. Each tube contains enough for one test.

Diagnostic and therapeutic sera are now available for all types of pneumococci, including Types I to XXXIII, with the exception that Types XXVI and XXX are no longer recognized as specific types and are considered identical with Types VI and XV respectively.

Group Typing:

Preliminary Grouping. To facilitate typing, a preliminary test is run with *Group* sera, the object being to determine in what group the organism belongs, after which the type in that group is determined. Group A contains antisera for Types I, II and VII. Group B contains antisera for Types III, IV, V, VI, and VIII. Group C contains antisera for Types IX, XII, XIV, XV and XVII. Group D contains antisera for Types X, XI, XIII, XX, XXII, XXIV. Group E contains antisera for Types XVI, XVIII, XIX, and XXVIII. Group F contains antisera for Types XXIII, XXV, XXVII, XXXI and XXXII.

Typing technic is the same as outlined. It is convenient to use two preparations on a slide. Some workers prefer to use coverslips and hollow ground slides (hanging drop method).

Arrange three slides as follows. Mark off division on each with glass marking pencil (Fig. 12.13).

1. Add one loopful of sputum and a drop of methylene blue.
2. Add 2 or 3 loopfuls or one capillary tube of group serum (Gr. A-F.).
3. Mix well with toothpick (one for each mixture) and cover with coverslip. Seal edges with Vaseline or other preparation.
4. Examine under immersion lens with partially diminished illumination. The reaction usually shows up in 2 to 5 minutes. If negative after this time, examine occasionally over a period of 30 minutes.

Final Typing. If any one *or more* Group serum-sputum mixtures show capsular swelling around the pneumococci, repeat the preparations outlined above, using the *Type* sera included in the Group serum mixture. For example: If typical capsular swelling was observed in Group A mixture, which contains *Types I, II, and VII* sera, separate tests on the sputum are run, each one with the separate *Type I, II,* and *VII* sera.

If more than one type is present, it is advisable to check with another sample of the sputum. Walter states that some indication of the causative agent may be gained from the relative proportion of the different organisms present and from the knowledge of the incidence of the different types of pneumococci present in healthy individuals. Types III, VI and XXIII are relatively more common in normal throats than are other types. Examination of a second specimen from the same case may reveal the presence of only one type. The type present in positive blood or spinal fluid cultures is always a causative type.

When the Neufeld technic is used with sputum containing many Type III organisms, the organisms may appear in large masses without definite capsular swelling. In such cases, it may be necessary to dilute the sputum with saline to obtain definite swelling of the capsule.

It has also been reported that Type III serum will sometimes cause agglutination of Type VIII organisms and will even give a Neufeld reaction with some Type VIII strains.

As previously stated, the method is also applicable to cultures, spinal fluid, pus, etc. (When cultures are used, the infinitesimal amount obtained on the tip of a straight wire is sufficient. A typical reaction will not develop if too many organisms are used.)

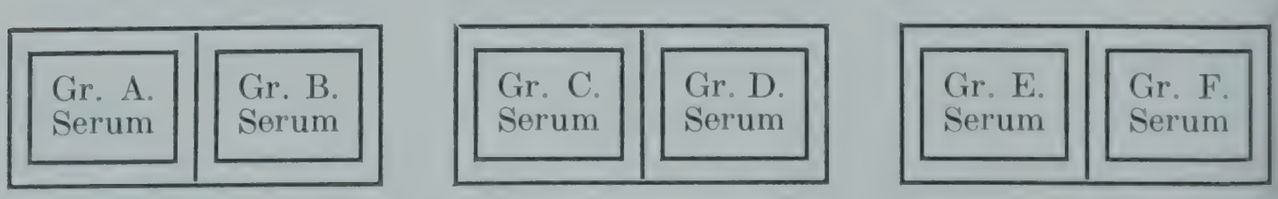


FIG. 12.13. Arrangement of slides for group typing

Typing of Pneumococci by the Mouse Method

When the pneumococci are not present in sufficient numbers for successful typing by the direct (Neufeld) method, animal inoculation is necessary. From 0.5 to 1 ml. of washed emulsified sputum is inoculated intraperitoneally into a white mouse (see fig. 12.14). From 6 to 24 hours later there will be sufficient growth of pneumococci in the peritoneal fluid for typing.

INTERPRETATION

Careful examination of the sputum and considered evaluation of the findings can be most helpful in the diagnosis and treatment of pulmonary disease. The specimen is generally easily obtained, the examinations simple for the most part, and the information gained frequently

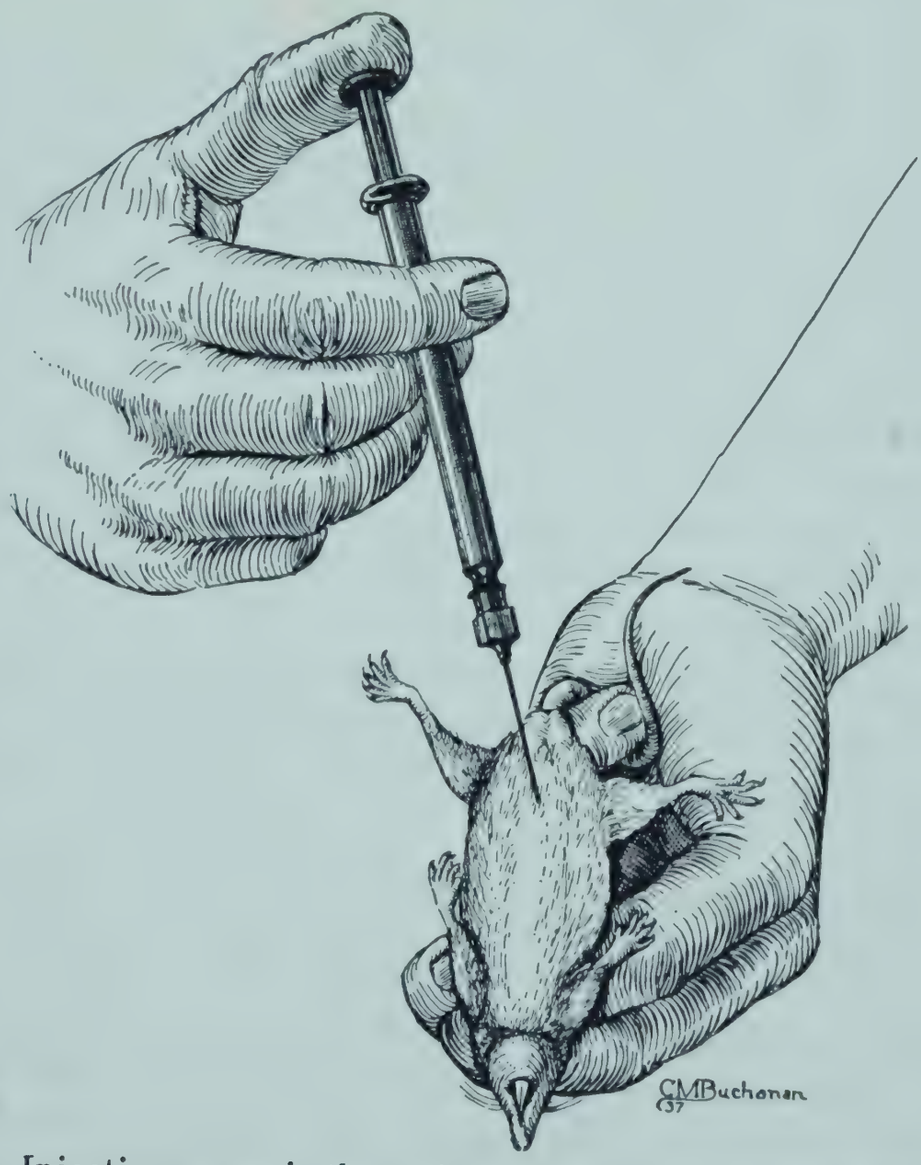


FIG. 12.14. Injecting mouse in the peritoneal cavity with pneumococcic sputum for typing. Note that the animal is held head downward so that the intestines will gravitate toward the head. It is held in the left hand by the back of the neck with the tail under the little finger.

most helpful. A negative result from a single examination is of little significance in ruling out any particular diagnosis, especially if the patient has received recent antibacterial therapy. Frequently repeated examinations are necessary, particularly in searching for *M. tuberculosis*. Sputum is generally contaminated by the saprophytic organisms from the throat and mouth and this must always be remembered in evaluating the results of a sputum examination. A sputum specimen should never be called negative for tubercle bacilli unless it has been examined by culture and/or guinea pig inoculation. *M. tuberculosis* usually shows macroscopic growth in 2 to 6 weeks in Lowenstein-Jensen or Petragnani's medium. Inoculated guinea pigs showing no disease should be held for 3 months and then autopsied. It is important to remember that if the patient is receiving isoniazid therapy, only culturing is useful for diagnosis because isoniazid may render the tubercle bacillus nonpathogenic for the guinea pig (Middlebrook *et al.*, 1953).

Many cases, largely children, have no sputum and cannot or will not cooperate in obtaining a satisfactory specimen. Here throat swabs and the flecks of material obtained from the accompanying coughing are readily obtained for examination. Such specimens are particularly important in acute pulmonary infections. However, the nasopharyngeal technic of Alexander *et al.* (1941) is preferred for culturing the nasopharyngeal mucus of children and is the method of choice for the diagnosis of whooping cough because this technic yields a greater percentage of positive cultures of *H. pertussis* than the cough plate method. Numerous individuals, particularly children, swallow their sputum and when tuberculosis is suspected in such individuals gastric washings should always be obtained.

Changes in the course of pulmonary diseases are frequently reflected early by changes in the amount and/or character of the sputum. Therefore it behooves the physician to carefully follow the sputum for such changes.

Bacteriologic Examination of Skin and Mucous Membranes

GENERAL CONSIDERATIONS

Exudates on mucous membranes and skin are generally purulent in character. They are most frequently caused by an infectious agent, the proper identification of which is the chief scope of examination of the exudate. Therefore study of the exudate by means of the various bacteriological, parasitological, mycological and virological methods represents the most important part of laboratory examinations as applied to skin and mucous membrane lesions.

However, it must be emphasized here that the recognition of the etiological factor behind the exudative process can not depend entirely upon the results of the bacteriological examinations. The surface of the skin and mucous membrane always contains many micro-organisms, some of which are definitely nonpathogenic, while others are facultatively pathogenic. The bacteriological examination of the exudate will therefore in most cases yield a multiplicity of bacteria and it will often be quite difficult, if not impossible, to recognize the micro-organisms responsible for the lesion. Secondary infection of skin lesions is a very frequent occurrence and often the progress of the disease is the result of more than a single type of micro-organism. In those instances the pathologist can only report the types of bacteria isolated from the exudate on the surface and the final evaluation must depend upon other clinical considerations.

COLLECTION OF MATERIAL

Certain general principles should be observed in obtaining and preparing material for examination. Purulent material that has remained on the superficial surface for some time is usually contaminated with various bacteria and may show certain degenerative changes that make it unfit for examination. Therefore the material should always be obtained as fresh as possible. The superficial purulent material should be removed and freshly exuding pus taken for examination. Sometimes it is necessary to apply a physiologic salt solution dressing on a cutaneous lesion for twenty-four hours to cleanse and moisten the surface. The material should be removed with a sterile wooden applicator, cotton swab, or inoculation wire loop. It also can be obtained by scraping the lesion gently with the sharp blade of a sterile scalpel or a small curette. Anesthesia of sensitive lesions with a 4 per cent sterile solution of cocaine may be necessary to permit deeper scraping of the surface. Small vesicles or abscesses should be cleansed with zephiran and punctured with a sterile needle and the contents aspirated with a tuberculin syringe. Since the results of bacteriological studies depend often upon the stage of the disease, the examination should be repeated several times at certain intervals. Any local treatment should be withheld for at least 24 hours before obtaining the material.

After the material has been obtained, some of it will be used for bacteriological culture or animal inoculation, while some of the exudate will be studied directly under the microscope. Smears may be made on slides by using a wooden applicator, cotton swab, or inoculation wire loop. Under some circumstances wet smears are preferable for exam-

ination as for fungi and Trichomonas, and, of course, are essential for dark field examination for spirochetes. The material should be carefully spread on slides so that it is thin and evenly distributed. Unevenly spread smears having thick and thin areas are very unsatisfactory for staining, especially by Gram's method, because of the impossibility of staining all parts uniformly. To prevent shrinkage of the cells smears should be dried rapidly by waving in the air, fanning, or with gentle heat which should not be greater than the hand can stand. See Chapter 13 for technics of examination for fungi if the latter is suspected.

Material from the eye should be obtained only from the diseased part and any contact with the healthy parts carefully avoided. In conjunctivitis the margins and angles of the lids should be avoided; in lacrimal gland infectious secretions should be obtained by expression. In corneal infections the cornea should be scraped, after previous anesthesia, with a sterile corneal knife. Pus from the anterior chamber of the eye can be aspirated with a fine needle and syringe.

Material from the ear should be collected by the specialist. The skin of the ear and the external auditory canal must be carefully cleansed with zephiran in order to avoid contamination.

Material from the nose may be obtained by blowing the secretion into a sterile gauze. Pus from the various sinuses should be obtained by the specialist. Cultures from the nasopharynx can best be made through the nose by using a stiff copper wire applicator with sterile cotton (Alexander *et al.*, 1941).

Exudates from the oral cavity and the pharynx are obtained with sterile cotton applicators or the platinum wire loop. The material should be collected only from the diseased area and contact with the normal oral mucosa should be carefully avoided. Sometimes it is advisable to probe into gingival pockets or tonsillar crypts. If much exudate is present it should be removed and the material taken from the underlying tissue surface. In case of suspected fungus infection the tissue must be scraped until it commences to bleed slightly.

One of the most important laboratory examinations is that of pathological exudates from the genito-urinary tract. The examinations indicated are to determine the presence, degree, and type of infection, including identification of the causative bacteria.

The examination in the male is usually that of free purulent discharge from acute urethritis. In chronic inflammatory disease the discharge may be scanty and only a small drop obtained on stripping the urethra or from prostatic massage. At times it is necessary to examine mucous threads that may be found in the urine.

In the female vaginal smears are very unsatisfactory for diagnostic examinations except in infants and young girls, because of the large number of bacteria in the normal vaginal canal. In acute lesions the causative organism may be found by deep scraping of the lesions, while in chronic infections a mixed bacterial flora is usually present. It should be borne in mind that the Bartholin ducts, the urethra and the cervix are much more often the site of pathogenic organisms than the vagina itself, and one should not fail to search those areas for infected exudates. To find *Trichomonas vaginalis* a fresh wet smear is made by placing a drop of normal salt solution on a slide and mixing material obtained on a swab or wire loop from the vagina.

PHYSICAL CHARACTERISTICS

The quantity of exudates from the skin and mucous membranes is usually not excessive. The material is nearly always turbid and varies in color between white, gray, yellow, red and black. A deep green color of pus is suggestive of pyocyaneus (*Ps. aeruginosa*) infection and is often found in the middle ear. The odor is usually not particularly characteristic except in the presence of putrefaction. Because of the large amount of fibrinolytic enzymes exudates usually do not coagulate. Only in certain types of infection does coagulation take place producing membrane-like structures covering the infected areas. Chronic lesions with development of granulation tissue tend to bleed easily and form dark colored crusts.

BACTERIOLOGIC EXAMINATION

The bacteriological examination of exudates from skin and mucous membranes represents one of the very important tests of clinical pathology. Here as in perhaps no other field can the clinical pathologist solve puzzling diagnostic problems and give pertinent advice with regard to the prognosis and therapy of the lesions. Two conditions must be fulfilled to make such a bacteriological study successful. The material must have been collected in the correct fashion with avoidance of all contamination, and the clinical pathologist must have a thorough knowledge of all phases of bacteriological diagnosis. The first condition has been discussed in detail in a preceding paragraph; for the second condition, which goes beyond the scope of this text, we refer the reader to the numerous books on bacteriological technic.

Generally speaking, two plans may be used to solve the problem of bacteriological diagnosis of a specific lesion of the skin and mucous membrane. One approach, which often promises the faster results

and which is therefore more widely used, attempts to confirm or disprove the most likely etiological diagnosis as suggested by the clinical appearance of the lesion. Actinomycosis, leprosy, tuberculosis or syphilitic lesions are infections which are usually suspected clinically and may be either confirmed or disproved by bacteriological examination. The other approach, which is the more tedious one, has to be used in lesions which do not suggest any probable etiology. After the bacteriological flora has been studied on the slide stained with Gram's method, on the wet preparation and under the dark field, the material should be grown aerobically and anaerobically on thioglycollate or trypticase soy broth, on blood agar plates and on Sabouraud's medium if fungi are suspected. Subcultures should be prepared as soon as small colonies appear and the various organisms should be properly identified by bacteriological methods. Animal inoculation may be tried, but in view of the frequent mass contamination of the lesions the results are not as good as with material obtained from the serous cavities or other "closed" lesions.

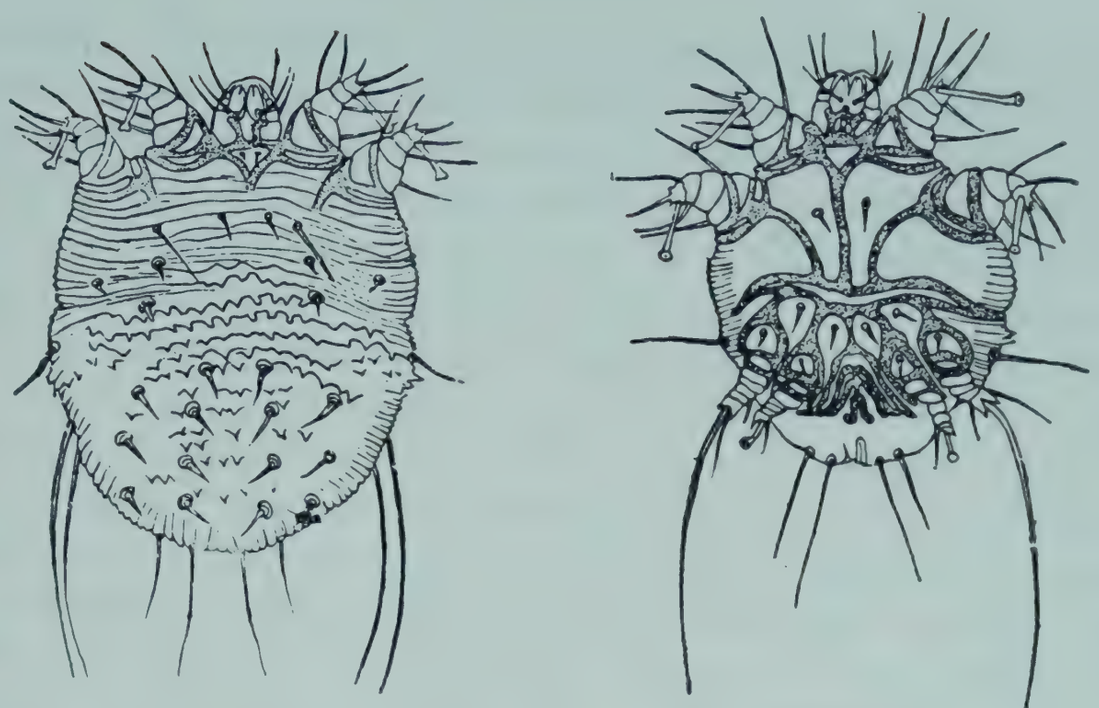
The following suggestions may be useful for material obtained from specific locations.

Skin

Pyogenic cocci and bacilli should be identified by culture on blood agar plates and carbohydrate broth. Tubercle bacillus may be grown on Petragnani or Löwenstein-Jensen medium. Spirochetes can be recognized under the dark field. The normal resident skin flora consists of diphtheroids, coagulase negative staphylococci, coliform bacilli, *Proteus sp.*, enterococci and *Pseudomonas sp.*

If fungus infection is suspected, material is placed on a slide in a few drops of 20 per cent potassium hydroxide and a coverglass applied. This is allowed to stand for thirty minutes, and then examined for spores and mycelia or budding forms. (See Chapter 13, Medical Mycology for further details.)

In examining for the itch mite, the parasite of scabies, the terminal end of burrows should be opened with a small scalpel, the material scraped out and mounted in water on a slide. The presence of the mite and ova are noted. With the low power objective the parasites are round or oval and set with bristles (Fig. 12.15). The males measure about 170 to 220 micra and the females about 300 to 400 micra. The ova are oval (120 x 75 micra) and may show the developing itch mite within the shell.



A B

FIG. 12.15. Sarcoptes scabiei: A. Female. B. Male, × 100. (Reproduced by permission from Medical Entomology, 2nd Edition, by Riley and Johannsen, Copyrighted, 1938, by McGraw-Hill Book Company, Inc.)

Eye

Most of the organisms can be identified by study of the smear stained with Gram's method.

Fresh purulent material obtained from the conjunctiva or cornea usually contains many neutrophilic leucocytes and some epithelial cells. Sometimes no bacteria are seen, or there may be an occasional organism from possible contamination. The definite presence of gonococci would indicate the cause of the infection.

The Koch-Week's bacillus (*Hemophilus conjunctividitis*), a small Gram negative bacillus closely resembling the influenza bacillus (*H. influenzae*) is the cause of acute epidemic conjunctivitis known as "pinkeye." These organisms are found usually in large numbers both extra- and intracellular.

The Morax-Axenfeld bacillus (*Hemophilus duplex*), a short thick, Gram negative Diplo-bacillus, sometimes occurring in short chains produces a subacute or chronic form of angular conjunctivitis, particularly marked in the area of the caruncle and outer angle of the eye.

Diphtheria bacilli may produce a pseudo-membranous conjunctivitis, though at times this may be produced by hemolytic streptococci. Other pathogenic organisms which may be found in eye infections are coagulase-positive staphylococci, pneumococci, *N. meningitidis*, alpha strep-

tococci, *Ps. aeruginosa*, *K. pneumoniae* and *Past. tularensis*. In addition to diphtheroids, other nonpathogenic organisms include coagulase negative staphylococci, gamma streptococci, *Gaffkya tetragena*, various nonpathogenic *Neisseria* and the colon-aerogenes group.

Cultures are required for the specific diagnosis of diphtheria bacillus infection since the *C. xerosis*, a nonpathogenic organism, is found frequently and cannot be distinguished on morphologic criteria.

Ear

Purulent material obtained immediately after perforation of the ear drum is of considerable value in determining the specific cause of otitis media. The bacteria usually found are pneumococci, hemolytic streptococci, staphylococci (coagulase positive), diphtheria bacilli, *H. influenzae*, Friedlander's bacilli, *Ps. aeruginosa* and colon bacilli. Typhoid bacilli may sometimes be found. Smears made later from a chronic discharging ear usually show contaminating bacteria and are of little value. Nonpathogenic bacteria commonly found in the ear are: coagulase negative staphylococci, gamma streptococci, *Gaffkya tetragena*, diphtheroids, *B. subtilis* and various *Neisseria*.

Nose

Smears and cultures from the nose made with an applicator cotton swab are of great value in the diagnosis of hemolytic streptococci, coagulase positive staphylococci, pneumococci and diphtheria. The pseudo-membranous exudate may not be present in the pharynx in some diphtheria patients.

Smears and cultures made from purulent material obtained with an applicator cotton swab from the opening of the antrum near the middle turbinate bone may be of value in determining the cause of infection of the sinus.

Probably the most satisfactory method for the diagnosis of leprosy is the finding of characteristic acid-fast lepra bacilli (*Mycobacterium leprae*) in smears or scrapings from the turbinate bones. Masses of these acid-fast lepra bacilli are found in what have been termed "lepra cells," but which have been more recently described as lymphatic thrombi.

Mouth and Throat

Depending on the cleanliness of the mouth, various numbers and types of bacteria are found including the saprophytic types in smears made from the mucous membranes without there being any definite evidence

of infection. Staphylococci (coagulase negative), streptococci, pneumococci, leptothrix, fusiform bacilli and spirochetes may be found in normal mouths that have not been recently cleansed. The most pertinent bacteriological problem in this location is the diagnosis of diphtheria or hemolytic streptococcus infection. When smears are taken directly from the lesion or when grown on culture media, diphtheria bacilli can be differentiated from most other bacilli with fair certainty. Examination of direct smears, however, presents more difficulty in recognition of diphtheria bacilli and should not be used except by experts. In cultures, however, the organisms have a characteristic morphology. They are rod-shaped organisms which vary in size from small to large bacilli. Many are club- or lanceolate shaped and show a swelling at one or both ends. Some are more or less banded so that some may appear as chains of streptococci. A variable proportion may contain one, two, or three deeper staining granules known as polar bodies. When only one or two are present they are usually situated near the ends. They may cause the bacilli to bulge resulting in a comma or dumbbell appearance. These characteristics are fairly well shown with Löffler's methylene blue stain, but are more pronounced with Ponder's special stain, where the bacilli are greenish blue with pink-red polar bodies. Occasionally bacilli are found which are morphologically typical but avirulent. These may be identified by isolation in pure culture and inoculating a guinea pig intraperitoneally (1 ml.) with a 48-hour broth culture. If the strain is virulent, the guinea pig should die within two days. A control guinea pig inoculated with the same amount of culture mixed with 500 units of diphtheria antitoxin should survive.

Vincent organisms can be demonstrated by staining the smears for two minutes with 2 per cent solution of crystal violet in methyl alcohol. Preponderance of the spirochetes is found in acute or active cases, while preponderance of the fusiform organisms speaks for a chronic or mild infection. The finding of Vincent organisms in smears from the gingiva or buccal mucosa should be considered significant only in the presence of pathological lesions.

Genito-urinary Tract

Inflammatory processes involving the kidneys, bladder and male sex organs can well be recognized by examination of smears prepared from catheterized urine, material obtained after prostatic message, or smears from urethral secretions. Colon bacilli, enterococci, alpha and beta streptococci and staphylococci (coagulase positive) are the most common

organisms present in pyelonephritis and cystitis. Urinary tuberculosis can be best diagnosed by inoculating urinary sediment into guinea pigs or culture media. Acute and chronic prostatitis will usually show pyogenic cocci and pus cells in smears obtained after prostatic massage. The gonococcus is the most common cause of acute inflammatory conditions of the urethra. When gonococci are not found on repeated examinations of urethral secretions, in spite of the presence of pus cells, the infection is usually referred to as "non-specific." In lesions of the genito-urinary tract, much of the bacteriological efforts are also directed to establish or exclude the presence of a veneral disease. The identification and culture of organisms which are the recognized agents of venereal infections will be discussed in detail in Chapter 18, Diagnosis of Venereal Lesions.

Bacteriologic Examination of Transudates and Exudates

DIRECT EXAMINATION

It is seldom necessary to examine transudates for bacteria but with all fluids suspected of being exudates it is essential that the centrifuged sediment be examined for bacteria. Some exudates, especially when markedly purulent, may be examined directly by placing some of the fluid on a slide, allowing to dry, fixing by heat or methyl alcohol, and staining. Bacteria may be seen in smears stained by Wright's stain but better results are obtained by use of Gram's stain. Smears stained with Wright's stain do not require previous fixation and in addition to staining the cells the bacteria are stained dark blue.

A properly stained Gram's smear shows the cellular elements including nuclei stained a red color by the safranin counterstain. The nuclei should not retain any of the crystal violet color; if so, the smear has not been sufficiently decolorized. Smears and cultures of exudates are usually positive for pneumococcus, streptococcus, tubercle bacilli, etc. As a rule transudates are sterile or are contaminated with skin staphylococci (coagulase-negative) or diphtheroids.

CULTURAL METHODS

Cultural methods are of value in the determination and the differentiation of various bacteria and fungi. The usual methods of staining and microscopic examination of material may show bacteria or fungi which cannot be identified, or may fail to show any organisms. Under such circumstances cultural methods are of great value. Relatively large amounts of fluid (1 ml. or more) should be cultured.

Tubes of thioglycollate medium, trypticase soy broth and blood agar plates, are used mainly in routine work, special media being used as indicated. The reliability of culture methods for tubercle bacilli has been improved greatly and should be used to a greater extent in searching for these organisms.

ANIMAL INOCULATION

Animal inoculation is of particular value for establishing the presence of tubercle bacilli (*Mycobacterium tuberculosis*) when they cannot be found by ordinary examination, the one disadvantage being the time required. Young healthy tuberculin negative guinea pigs should be used. The material for injection should be obtained in such manner that coagulation is prevented. If possible collect several ounces of fluid for concentration. If coagulated material has been obtained it should be liquefied by the addition of normal sodium hydroxide solution and incubation at 39°C. for 30 minutes. Neutralize with an equal amount of N/1 HCl and centrifuge the fluid at high speed. The residue is then injected into one and preferably two guinea pigs.

If the alkali has not been used, after centrifuging and pipetting off supernatant fluid the sediment is mixed with 1 or 2 ml. of normal salt solution drawn into a sterile syringe and an equal portion injected into each groin of a guinea pig (Fig. 12.16). Gentle massage of the injected

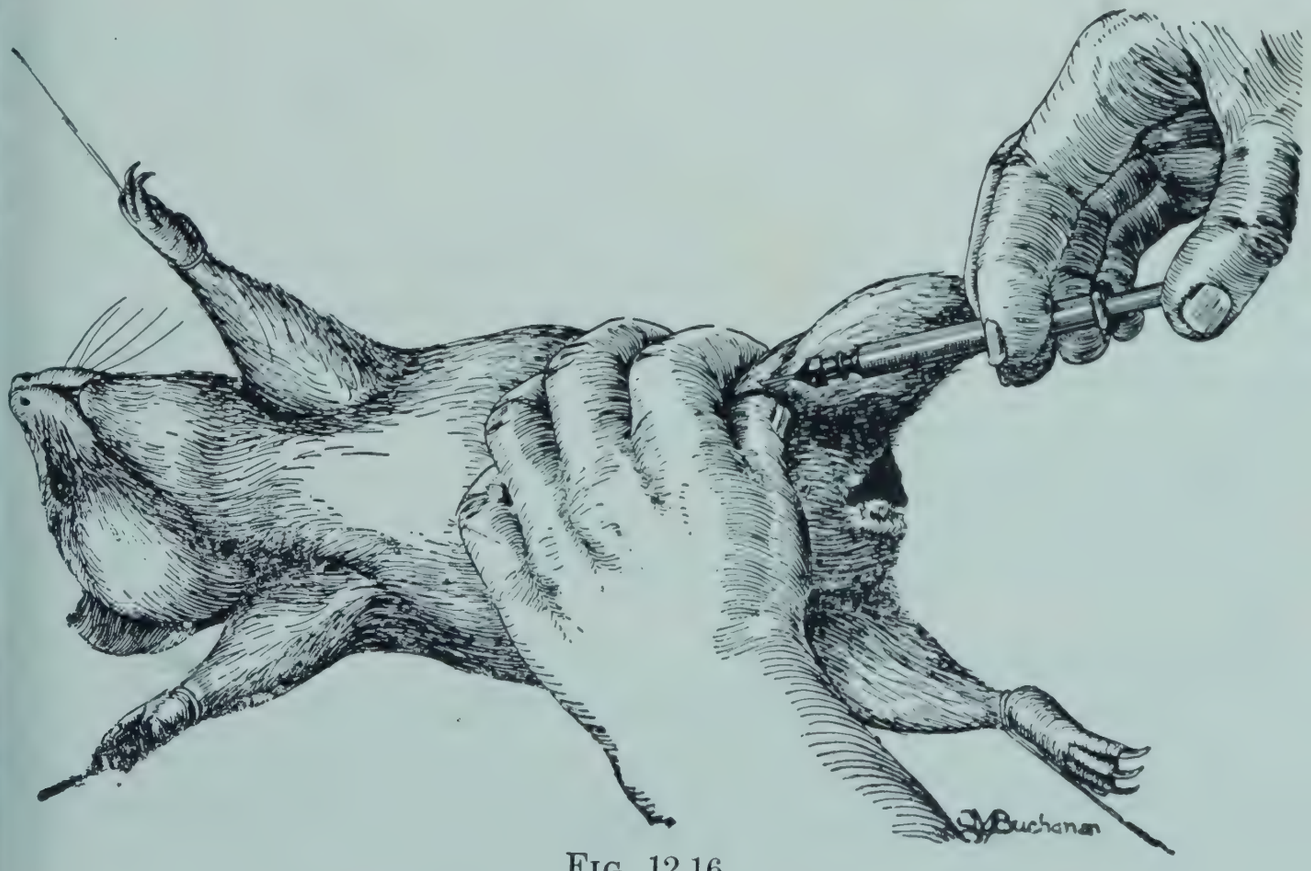


FIG. 12.16

area for one or two minutes aids lymphatic absorption. If the fluid is thought to be free of other bacteria some of it may be injected directly into the peritoneal cavity of the same or another pig.

When the animal dies or is killed at the end of six weeks a careful examination is made of the inguinal, posterior mesenteric, and peribronchial glands, as well as the spleen, liver, and lungs, for tuberculosis. These tissues should be examined by acid-fast stains of the fresh material and by histologic sections.

CLINICAL CORRELATION

Pericardial Effusions

A thick fibrinous plastic exudate may be seen in acute pericarditis of rheumatic fever. Purulent pericardial exudates are found most frequently in pneumococcic or staphylococcic (coagulase-positive) infections. In such cases the amount of effusion may be quite large. Pericardial effusions may be hemorrhagic in tuberculosis or hemolytic streptococcic infection.

Pleural Effusions

Serofibrinous effusions occur most frequently in pulmonary tuberculosis. Tubercle bacilli are usually absent in the centrifuged sediment, but often can be found by concentration methods in culture or with animal inoculation.

The character of the fluid in empyema (pleuritis) is determined by the causative bacteria. In pneumococcic infections the fluid is usually yellowish or greenish in color, thick and creamy. In streptococcic infections, usually of the hemolytic type, it is more seropurulent in character. In tuberculous empyema it may contain caseous masses in a rather thin purulent fluid. Other bacteria which may be isolated from empyema pus include coagulase positive *Staph. aureus* or *H. influenzae*. The pus from rupture of an abscess or gangrene of the lung is often putrid, having a foul odor.

Hemorrhagic fluids, particularly those of dark brownish appearance may be found in fluids of pulmonary tuberculosis and rarely in other diseases.

Peritoneal Effusions

In tuberculous peritonitis the fluid is usually thin and slightly cloudy, contains an increased protein content and has a specific gravity of above

1.020. Tubercle bacilli are rarely found in the fluid except by concentration methods and culture or guinea pig inoculation. In peritonitis from colon bacillus infection the purulent fluid usually has a fecal odor. This characteristic is also noted after perforation of the intestine. In acute inflammatory conditions in which there has been no escape of intestinal organisms into the peritoneal cavity there may be a cloudy serous fluid, free of bacteria, containing small flakes of fibrin that may appear to be pus. This fluid, while having an increase in the total number of leucocytes, is not considered as purulent. Pus obtained from the abdominal cavity may show, in addition to degenerated leucocytes, numerous types of bacteria of which the colon bacillus is most common. The hemolytic streptococcus, enterococcus, pneumococcus, coagulase-positive staphylococcus, *Cl. perfringens*, Bacteroides, Paracolon bacilli, *S. typhi*, *Ps. aeroginosa* and gonococcus may be found in occasional instances.

Synovial Fluid

The amount of synovial fluid obtained usually amounts to only a few cubic centimeters. The presence of numerous neutrophils characterizes a purulent arthritis. Bacteria may be found in acute purulent conditions either by smear or culture and tubercle bacilli by culture or guinea pig inoculation. The organisms most frequently sought are gonococci, but they are found rarely on examination of direct smears. Cultures in chocolate blood agar incubated in an atmosphere of 10 per cent CO_2 offer better results.

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CHAPTER 13

MEDICAL MYCOLOGY

By Emma S. Moss and Albert L. McQuown

Fungous diseases are world wide in distribution. Though infections with some species show a higher incidence in one or another geographic location, this factor is losing much of its importance due to increased travel by means of more rapid transportation.

There is an increasing awareness of the importance of infections caused by fungi, particularly those producing systemic disease. Long considered rare, or at least infrequent, we know that many cases have gone undiagnosed or have been misdiagnosed.

The clinician who first sees the patient should be alert to the possibility that any given disease may be due to fungi, and so consider it either as a primary diagnosis or in the differential diagnosis.

Fungous infections are seldom clear cut in their clinical manifestations. Systemic infections by a number of genera simulate each other as well as other diseases such as tuberculosis, various granulomas, lymphomas, and other malignancies.

Improved methodology in the diagnostic laboratories has led to isolation and identification of species of fungi in many otherwise obscure cases.

Accurate diagnosis can be accomplished only in the laboratory; however, a presumptive diagnosis can often be made on history, physical examination and the findings of suspicious forms in exudates, body fluids or scrapings from the lesion.

Definitive diagnosis is imperative as treatment varies. With more successful methods of treatment, prognosis is greatly improved—especially in some of the more severe infections which were formerly considered invariably fatal.

For practical purposes the medically important fungi may be divided into three broad classes depending on the type of disease most frequently produced.

Systemic mycoses: Actinomycosis, nocardiosis, cryptococcosis, moniliasis, blastomycosis (North American type), blastomycosis (South American type), histoplasmosis, coccidioidomycosis, occasionally mucormycosis, aspergillosis and geotrichosis are generally systemic infections affecting various organs and systems.

TABLE 13.1

	Tissue Involved				Forms Seen in Exudate or Scrapings									
					Buds				Spores					
	Systemic	Cutaneous	Mucous membranes	Central nervous system	Reticulo-endothelial system	Single	Multiple	Capsule	Single	Spherule with endospores	Hyphae	Granules	Clubbing of hyphae	Acid-fast
A. israeli (bovis)	x	x									x	x	x	
N. asteroides	x										x	x		x
Nocardia sp.	x	x									x	x		±
C. neoformans	x	x		x		x		x						
C. albicans	x	x	x	x		x								
B. dermatitidis	x	x				x								
B. brasiliensis	x	x				x	x							
H. capsulatum	x	x	x		x	x								
S. schenckii	x	x												
C. immitis	x	x							x	x				
H. pedrosoi		x							x					
H. compactum		x							x					
P. verrucosa		x							x					
Trichophyton		x							x		x			
Microsporum		x							x		x			
Epidermophyton		x							x		x			
N. tenuis		x									x			
N. minutissima		x									x			
M. furfur		x							x		x			
P. hortai		x							x		x			
T. beigelli		x									x			

Intermediate mycoses: Moniliasis, sporotrichosis and chromomycosis are generally infections of skin, mucous membranes and subcutaneous tissues; although they may occasionally become systemic.

Superficial mycoses: These are superficial infections of hair, skin and nails caused by the dermatophytes and sometimes *Candida*.

ACTINOMYCOSIS

Disease. Actinomycosis may take any one of several clinical forms. It may be cervico-facial, muco-cutaneous, thoracic, abdominal or gen-

eralized. Clinical symptoms depend upon the location of the lesions and duration of infection.

Cervico-facial infection is the most frequent form of the disease. This *usually* follows trauma to the mouth such as tooth extraction or long standing poor oral hygiene. During the early stage of the infection there is *usually* painless swelling with relatively little inflammatory reaction. After some time the tissues break down and form draining sinuses. The other forms of the disease may result from aspiration or swallowing of infected material or by direct extension. Likewise, in these forms there is "woody" tumefaction which later breaks down and leads to formation of draining sinuses. The abdominal type often simulates acute or subacute appendicitis or salpingitis. This type is frequently not suspected until found at operation. Pulmonary actinomycosis tends to involve the pleura and produce characteristic pleurocutaneous sinuses.

Diagnosis. The etiologic agent of actinomycosis is *Actinomyces israeli* (*bovis*). This organism characteristically produces so-called "sulfur granules" consisting of intertwining mycelium which often has clubs surrounding the periphery of the granule. The granules are yellowish in color and usually can be picked out of the pus or exudate from the incised or aspirated lesion or material from the draining sinus. These may be recognized in wet, unstained preparation or the crushed granule may be stained by Gram's method. Following Gram's stain the central part of the colony stains Gram positive. The clubs represent body reaction to the organism and are not part of the intertwining mycelium. Occasionally complete granules are absent but the presence of Gram positive branching filaments (1 μ in diameter) should make one suspicious of the presence of *Actinomycetes*. For the positive diagnosis of the species, exudate from draining sinuses, pus, sputum or excised tissue should be submitted to a laboratory equipped to culture the exudate on suitable media under anaerobic or micro-aerophilic conditions.

Positive diagnosis of actinomycosis can be made by the detection of the granules on wet or stained preparation which may be confirmed by culture in the laboratory if material obtained by aspiration or excision of the lesion is uncontaminated by bacteria. Growth usually occurs within 5 to 7 days. Nocardiosis must be considered if only Gram-positive branching filaments are observed in the stained material and there is bacterial contamination. The diagnosis must be proved by definitive culture to rule out bacteria as the etiologic agent with particular reference to *Mycobacterium tuberculosis*.

Pus from suspected lesions should be aspirated under sterile conditions where possible. Pus should be collected in a test tube or it may be possible to obtain material by compression of the surrounding undermined tissue. It may be necessary to irrigate the sinus tract and curette the wall if no exudate can be obtained otherwise. Occasionally sufficient exudate can be obtained by placing a pack of saline-moistened gauze over the sinus overnight. Sputum is occasionally suitable for diagnosis by culture; however, it is difficult to obtain growth from such contaminated material and the non-pathogenic *Actinomycetes* must be ruled out. In order to detect "sulfur granules" the material obtained should be placed in a test tube or petri dish and rolled about the container by tilting while observing under a bright light. Suspected granules should be taken from the material and placed on a slide with a small amount of saline, covered with a cover slip and gently crushed. The granule will be semi-opaque, yellowish, lobulated and composed of innumerable branching filaments tending to radiate to the periphery (ray-fungus) (Fig. 13.1). Many of the granules may have hyalin-like clubs at the periphery produced by body reaction; however, this is not always present. The granule should be stained by Gram's method for the characteristic picture. If granules are not present, representative material should be placed on a slide, dried and stained by Gram's method to search for the Gram-positive branching filaments.

Interpretation. Actinomycosis must be differentiated from tuberculosis and other infectious granulomas, other mycotic diseases such as nocardiosis, coccidioidomycosis, blastomycosis, cryptococcosis and moniliasis, chronic abscesses due to certain bacterial agents, and benign and malignant tumors especially those affecting lymph nodes, bones and soft tissue.

The presence of "sulfur granules" in wet preparation or stained smear confirmed by culture in the mycological laboratory leads to a positive diagnosis of actinomycosis.

NOCARDIOSIS

Disease. Nocardiosis may be pulmonary, systemic or may produce mycetomas of various parts of the body, most frequently of the lower extremities (mycetoma pedis). The acid-fast species, *Nocardia asteroides*, as well as several other species which are non-acid-fast may be the etiologic agent. The characteristic lesion is suppurative in nature rather than granulomatous. Nocardiosis as a pulmonary and systemic entity has only been appreciated within the past few years. The acute dif-

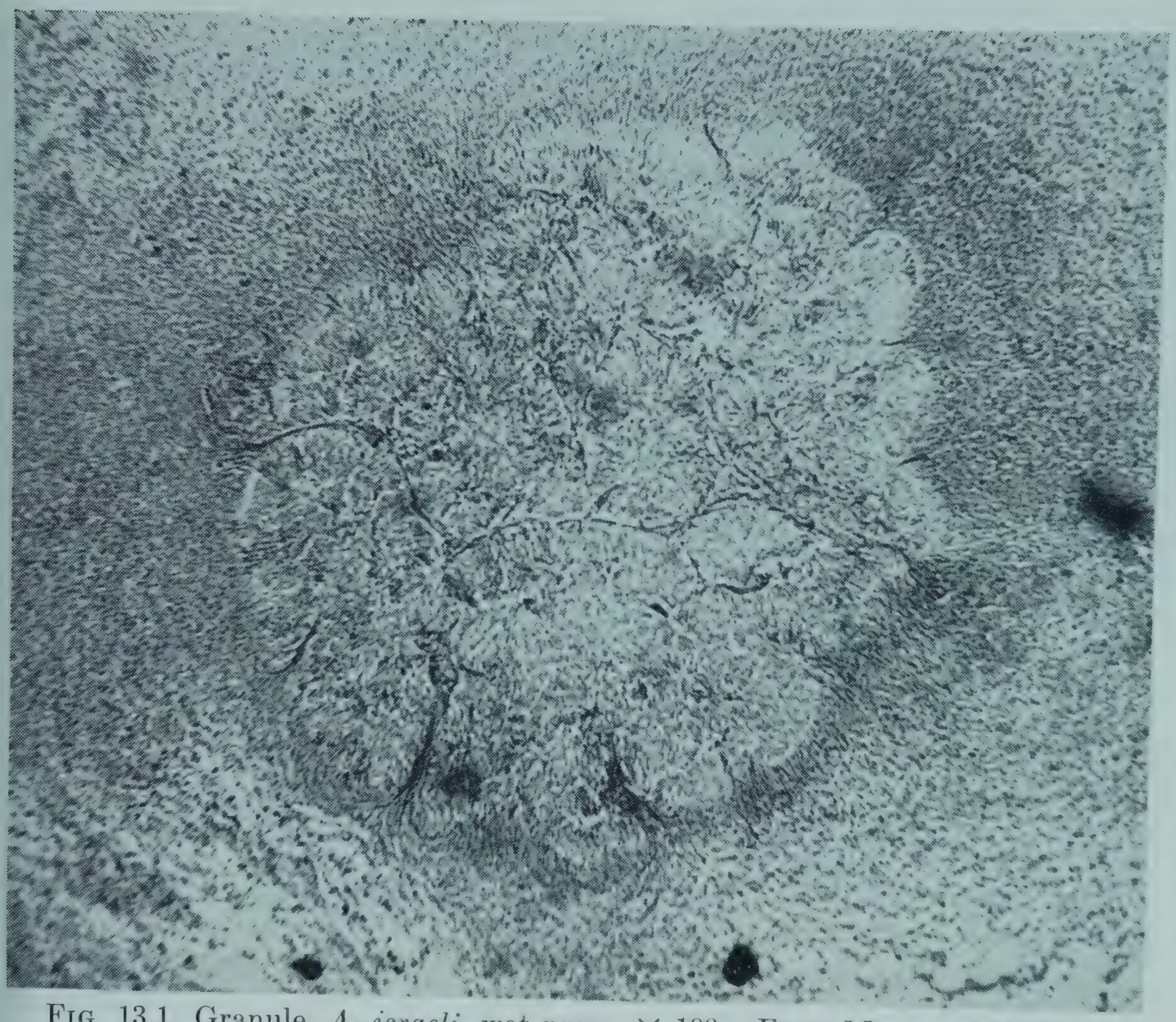


FIG. 13.1. Granule, *A. israeli*, wet prep., × 100. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

fused lobular type pneumonitis and bronchitis produced by *Nocardia* have usually been attributed to bacterial agents. The more chronic types of pulmonary disease have with some frequency been confused with tuberculosis. The confusion with tuberculosis may be four-fold: (1) The clinical signs and symptoms are not unlike those of tuberculosis; (2) *Nocardia asteroides* as well as some of the other species are acid-fast when stained by the Ziehl-Neelsen method; (3) *Nocardia* species grow on most of the culture media recommended for the cultivation of *Mycobacterium tuberculosis* and often are indistinguishable morphologically from colonies of this organism; (4) When inoculated into guinea pigs as a confirmatory test for pathogenicity, both *M. tuberculosis* and *N. asteroides* produce lesions in the animals. Other species of *Nocardia* may be non-pathogenic under these circumstances. The neutral red test for virulence of *M. tuberculosis* hominis may be useful in differentiation, as *N. asteroides* as well as a number of other species that have been

studied by this test have proved to be neutral red negative while *M. tuberculosis hominis* is neutral red positive.

Diagnosis. Unlike *Actinomyces israeli*, species of *Nocardia* rarely produce identifiable granules. Those granules produced are looser in make-up and clubbing is absent. Sputum, bronchogenic washings, pus or other exudate should be collected under as aseptic conditions as possible. Search should be made for granules. If these are not present, smears should be made on clean slides and stained by Gram's method and by an acid-fast method taking care not to over decolorize. Examination of the Gram's stained smear will reveal gram-positive branching and bacillary forms measuring 1 μ in width. The exudate is purulent. Acid-fast forms may or may not show branching and when branching is not present the organisms are indistinguishable from *M. tuberculosis*. Suspected sputum and other exudate should be submitted to the mycological laboratory for definitive culture and confirmation of the diagnosis. If acid-fast branching filaments are identified the organism is *Nocardia asteroides* or other species of acid-fast *Nocardia*. If the branching gram-positive filaments are not acid-fast the specific etiologic agent must be differentiated by culture (Fig. 13.2).

Interpretation. The presence of gram-positive branching filaments or a loosely formed filamentous granule should lead one to suspect nocardiosis and suitable material should be submitted to the laboratory for culture. The presence of acid-fast bacillary forms should suggest nocardiosis as well as tuberculosis and specific measures taken to prove the etiologic agent producing the disease.

CRYPTOCOCCOSIS

Disease. Cryptococcus is caused by the single pathogenic species *Cryptococcus neoformans* (*Torula histolytica*). This yeast is a single or budding, round or oval organism that measures 5 to 18 μ in diameter. Generally the organism is surrounded by a wide, transparent gelatinous capsule and there is an absence of pseudo-hyphae or hyphae.

A chronic cerebro-spinal meningitis is the most frequently recognized form of cryptococcosis. The disease is insidious and symptoms develop gradually, beginning with headache and vague symptoms of central nervous system involvement. Later the symptoms seem to be exaggerated for the physical condition of the patient, who does not appear acutely ill. Terminally the patient's symptoms and physical findings are those of a severe chronic meningeal infection. Pulmonary forms of the disease initially produce a mild pneumonitis productive of a small

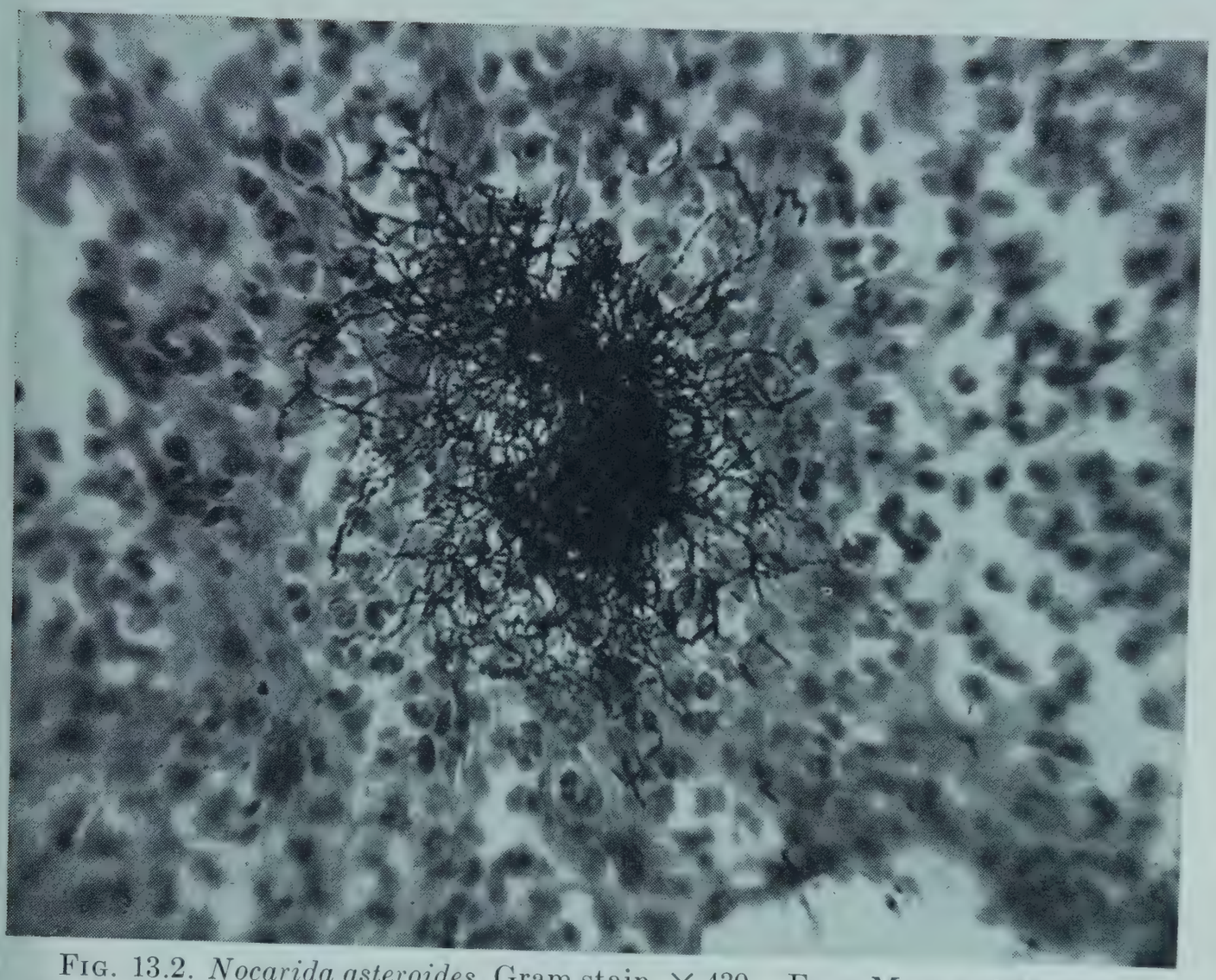


FIG. 13.2. *Nocarida asteroides*, Gram stain, × 430. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

amount of mucoid bloody sputum. Later the lung lesions are diffuse and a large amount of mucoid sputum is produced. Skin and mucocutaneous lesions are vesiculo-papular with gradual extension and ulceration. Systemic forms of the disease may produce lesions in the brain, lungs, kidneys and other organs.

Diagnosis. Spinal fluid should be collected aseptically. Physical and chemical properties are similar to those found in tuberculous meningitis. Care should be taken in counting the cells in the spinal fluid, as the small forms of *C. neoformans* are approximately the size of lymphocytes and may be mistaken for them. Examination under reduced light will usually reveal the organism to be surrounded by a wide gelatinous capsule (Fig. 13.3). This capsule can be demonstrated easily by mixing a small amount of India ink or stain with the spinal fluid and examining immediately (Fig. 13.4). It may be necessary to centrifuge the spinal fluid to concentrate the few organisms present. Material aspirated from lesions as well as scrapings from ulcers and sputum

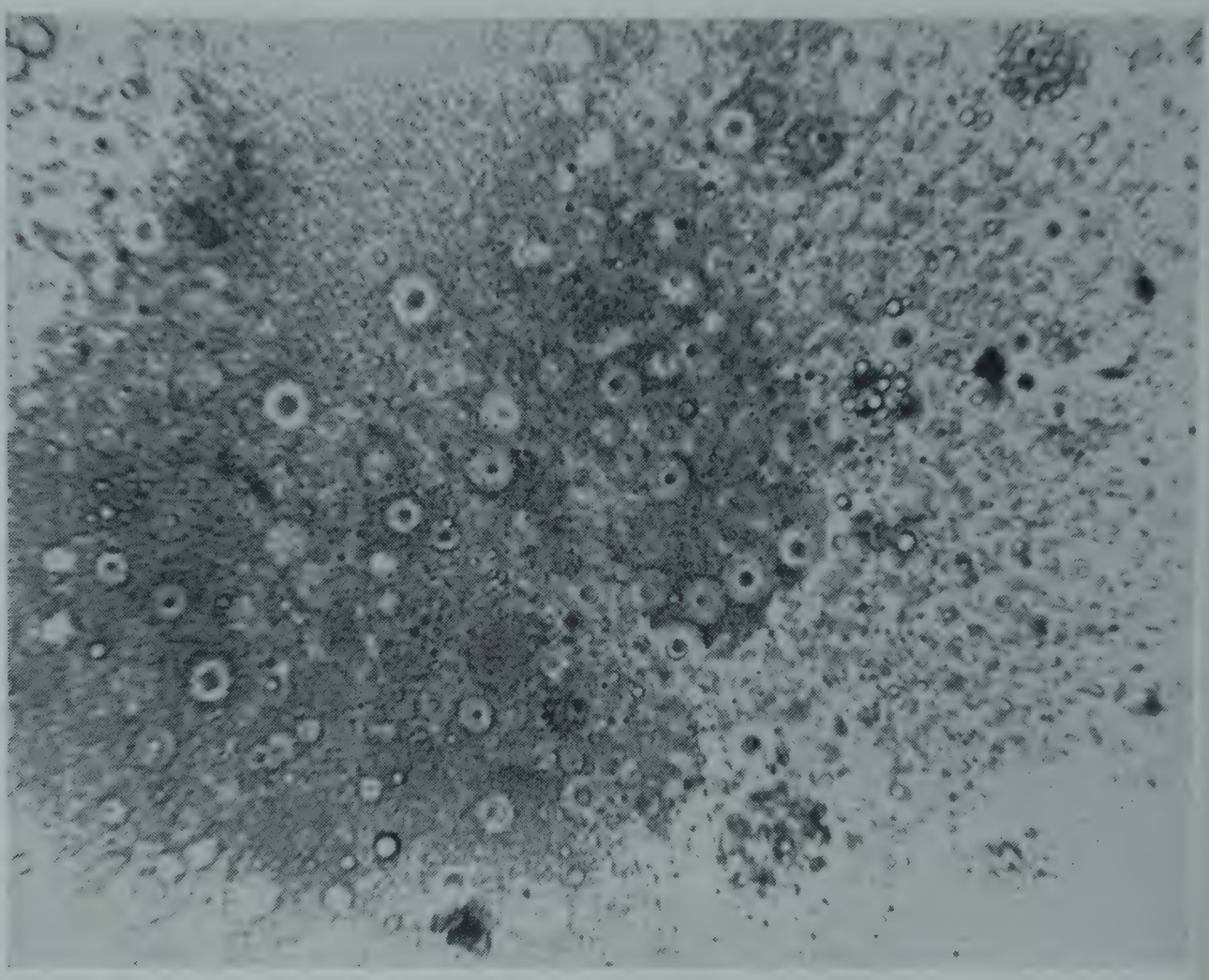


FIG. 13.3. *C. neoformans* in pus, × 410

should be examined carefully for the round, single budding yeast cells which are surrounded by typical wide gelatinous capsules. Material must be submitted to the laboratory for definitive culture and identification.

Interpretation. The presence of single budding yeast cells surrounded by large gelatinous capsules should lead to a diagnosis of cryptococcus meningitis. The spinal fluid of a meningitis case that does not yield a specific bacterial agent should be searched carefully for this organism and the cultures held for a longer length of time as the incubation period of fungi is much longer than the pyogenic bacteria.

If numbers of budding and non-budding yeast are present in sputum or exudate and no other likely etiologic agent is demonstrated, cryptococcosis should be suspected and cultures to demonstrate the presence of *Cryptococcus neoformans* should be requested.

MONILIASIS

Disease. Moniliasis may be a localized or a generalized disease, usually produced by the organism *Canida albicans* but occasionally by

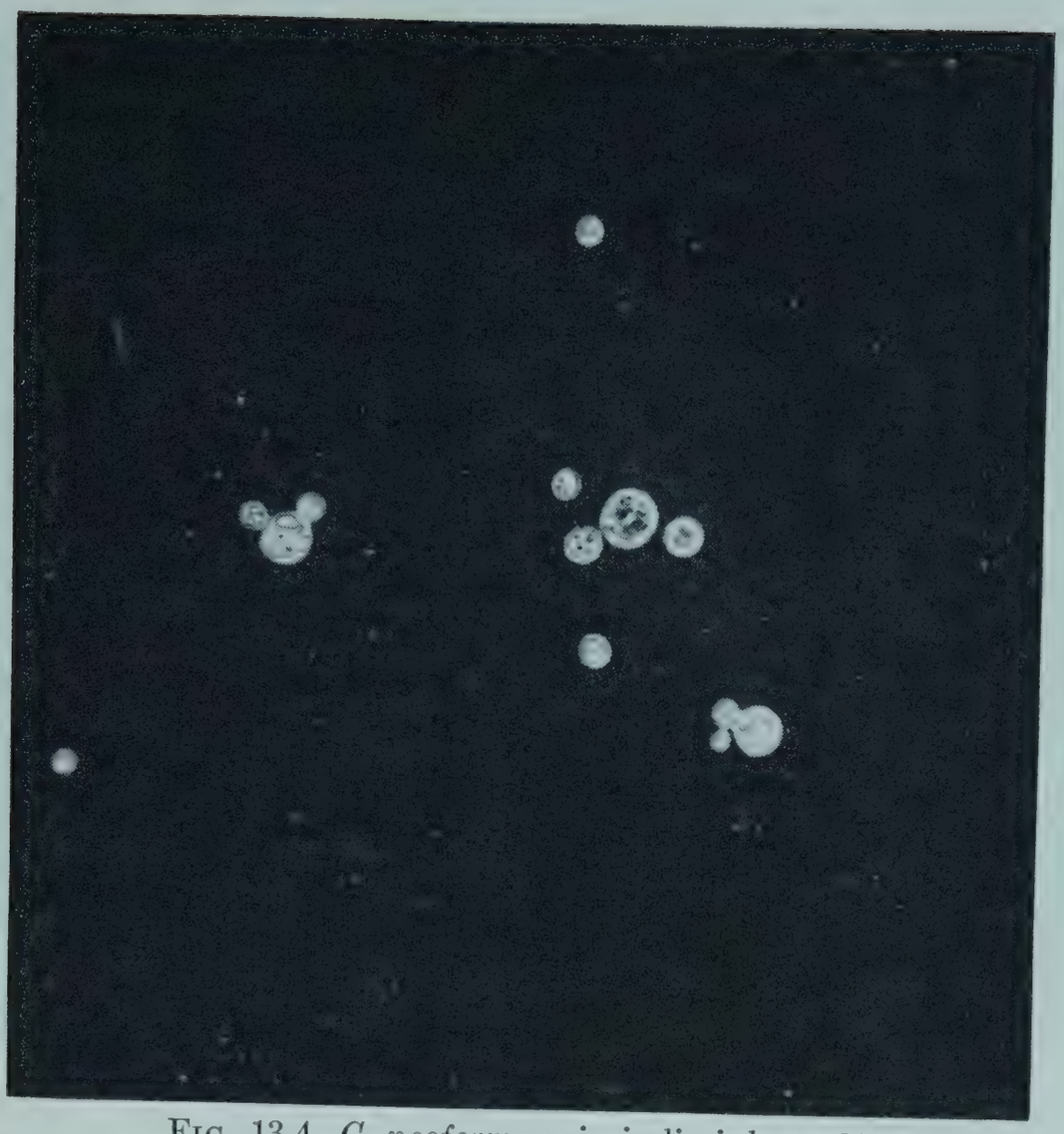


FIG. 13.4. *C. neoformans* in india ink, × 330

other species of *Candida*. Localized infections may be cutaneous, involving the nails and cuticle or affecting the oral mucosa to cause thrush, glossitis and perleche. Infections of the vulva and vagina by this organism are frequent complications of pregnancy and debilitating diseases. Generalized infections may be cutaneous or systemic. Lesions in systemic moniliasis are most frequently found in the lungs, meninges, pericardium and epicardium, although occasionally other organs may be involved. With widespread use of broad-range antibiotic therapy, generalized and often fatal infections due to these species are not infrequent. Species of *Candida* are present in the sputum and feces of normal individuals, sometimes in large numbers, indicating that the majority of Candida infections are endogenous.

Diagnosis. Scrapings of skin and nails should be placed on a slide, covered with 10 per cent KOH, heated gently to clear the epithelium and covered with a cover slip. The organisms are present as small round or oval budding yeast-like (2 to 4 μ) cells, and if pseudo-hyphae are present the cells may be attached at the point of constriction (Fig. 13.5



FIG. 13.5. *C. albicans*, wet preparation (sputum), × 100

Sputum and material from the mouth and vagina should be mixed in saline, covered with a cover slip and examined for yeast-like organisms (Fig. 13.6).

The presence of the above described organisms shows the presence of *Candida* species or other yeast-like species and the material must be submitted to the laboratory for the definite identification of Candida species and further culture for specific identification as *Candida albicans*.

Interpretation. In superficial infections, such as thrush or vaginitis, the finding of numerous yeast-like organisms is fairly diagnostic of moniliasis—although the diagnosis should be confirmed by laboratory culture. In the systemic diseases and more generalized infections, the mere presence of yeast-like organisms even in large numbers in sputum and stools is not diagnostic of moniliasis unless the organisms are recovered on numerous occasions, proved by culture to be *Candida albicans*, and no other etiologic agent can be demonstrated.

Skin tests, agglutination and serological tests are not of diagnostic significance and are generally performed only in the research laboratories.

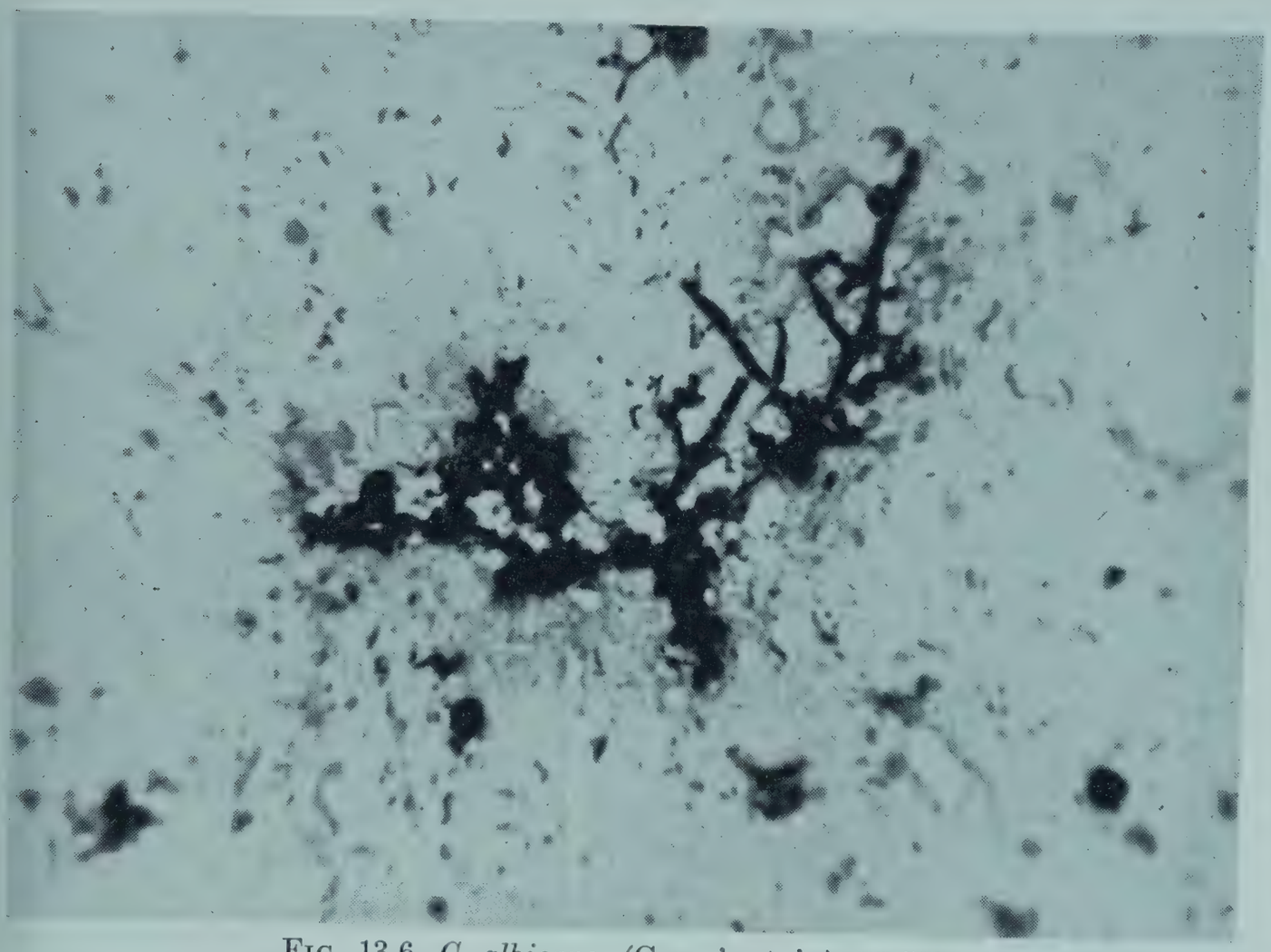


FIG. 13.6. *C. albicans*, (Gram's stain), × 500.

NORTH AMERICAN BLASTOMYCOSIS

Disease. Blastomycosis, North American type, is caused by *Blastomyces dermatitidis* and may be either cutaneous, pulmonary or systemic. The cutaneous lesions are first maculo-papular in character, later becoming pustular. They spread from the periphery, tend to heal in the central portion, and the raised crusted edge may show numerous microabscesses present. The patient is most frequently a male in the middle age group. The primary site is usually the extremities or the face. The history of injury or association with vegetation generally precedes the infection. Occasionally cutaneous lesions spread to produce systemic infections, although more usually the systemic infections are primary in the lungs. The pneumonitis is chronic, and may result in abscess formation, or may become generalized with spread to adjacent organs such as the pericardium, bones, diaphragm. Blood-tinged sputum containing the organisms may be scant or produced in large quantities.

Diagnosis. Material from the cutaneous lesions may be obtained by

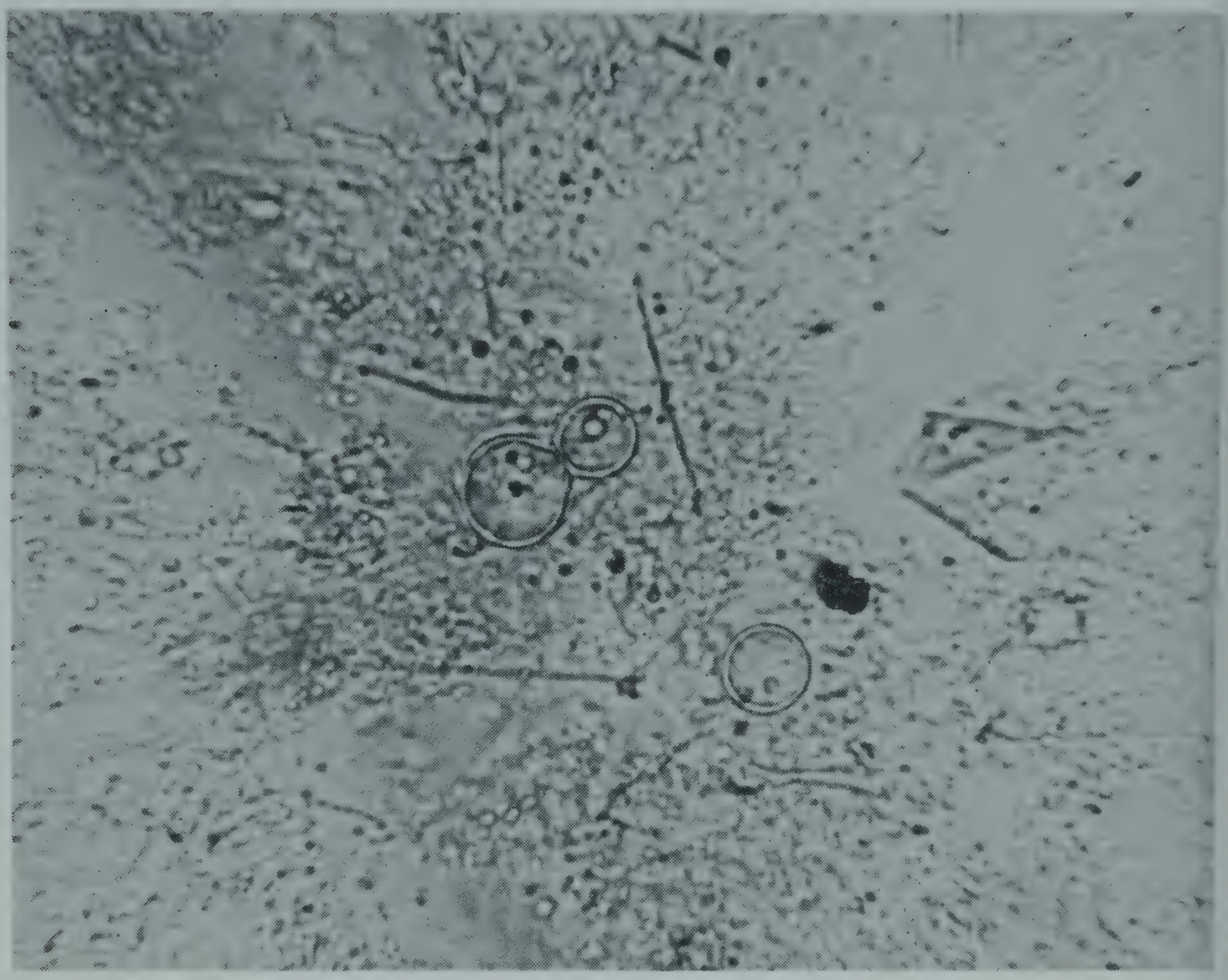


FIG. 13.7. *Blastomyces dermatitidis* in pus, × 500

gently opening one of the micro-abscesses at the periphery of the lesion and examining the material thus obtained for the presence of blastomycetes for diagnosis. These organisms appear in the inflammatory exudate as thick-walled, double contoured, refractile, single or single budding, round or oval yeast cells. They measure from 8 to 15 μ in diameter (Fig. 13.7). Occasionally they are present in large numbers but at other times they are few and must be searched for diligently.

Sputum should be examined unstained in wet preparation. Morphologically *Blastomyces* are the same as in material from the micro-abscesses. Because of considerable variation in size and resemblance to other yeasts, suspected material must be submitted to the mycological laboratory for culture and identification.

Skin tests with Blastomycin and complement fixation tests may give presumptive evidence of Blastomycosis when this infection is suspected, but the diagnosis should be confirmed by demonstrating the organisms in exudate and on culture. When the diagnosis has been proven by culture these tests are valuable in evaluating the prognosis of individual cases.

Interpretation. Skin tests using blastomycin should be performed. The test is usually positive if the patient has or has had the disease. It may be negative in advanced cases where anergy has developed. There is cross immunity between blastomycosis and histoplasmosis; therefore, the patient should be skin tested with both these antigens at the same time. The blastomycin test will be the stronger, giving a typical reaction in 24 to 48 hours, and in very sensitive individuals may lead to abscess formation in blastomycosis.

Complement fixation titers are generally low, but may be high in severe systemic disease. The presence of a high complement fixation titer and a negative skin test denotes a poor prognosis, while a low or absent complement fixation titer and a positive skin test usually indicates a good prognosis.

The positive diagnosis of Blastomycosis, North American type, is made only when the organism *Blastomyces dermatitidis* is cultured from the patient.

SOUTH AMERICAN BLASTOMYCOSIS

Disease. Blastomycosis, South American type, is produced by *Blastomyces brasiliensis*, and for practical purposes is limited to South America. There are several forms of the disease: cutaneous, lymphangitic, visceral and a mixed type. The lesions are similar to those granulomas produced by *Blastomyces dermatitidis*. The disease has never been reported from North America but is included here for differential diagnosis, since travel between the two continents is increasing rapidly.

Diagnosis. Material should be obtained by aspiration or scraping the suspected lesion and examining the material in wet preparation. The organism is a blastomycete measuring 10 to 60 μ in diamter. It appears as a single, or multiple budding, thick-walled organism. The diagnostic form is the larger cell, showing numerous smaller buds around the periphery. Material should be submitted to the laboratory for culture and definitive diagnosis.

Interpretation. South American blastomycosis is diagnosed by culturing *Blastomyces brasiliensis* from the lesions. There is cross immunity between *Blastomyces brasiliensis* and *Blastomyces dermatitidis*. Skin tests and complement fixation tests done on cases produced by *Blastomyces brasiliensis* are interpreted as discussed in the section on North American blastomycosis.

HISTOPLASMOSIS

Disease. Histoplasmosis is caused by *Histoplasma capsulatum*. It may be acute, sub-acute or chronic, localized or disseminated, primarily involving the reticulo-endothelial system. The initial lesion may be a shallow ulcer of the skin or mucous membrane or it may be a primary pulmonary disease caused by inspiration of dust containing the organisms. The latter cases tend to be in persons exposed to infected material while working in caves, silos, and barns. The majority of cases are self limited, similar to the infectious complex of primary tuberculosis which produces the Ghon lesion. Occasionally the disease will spread from the focal area and involve the entire reticulo-endothelial system, producing lymphadenopathy, splenomegaly and hepatomegaly. In this generalized form, the course is fulminating. If the spread is to a few areas the disease may be confused with or erroneously diagnosed as malignant lymphoma. Pulmonary involvement is generally bilateral; the lesions are more frequently found in the bases. Calcified lesions resembling tuberculosis are frequent and may be numerous. Cavities are occasionally found.

Diagnosis. Scrapings from suspected ulcers should be made into wet preparations and examined. In disseminated cases the blood, bone marrow, material from lymph nodes, sputum, urine and even stools should be examined and cultured. The organisms found in exudates and tissues are small, round, or oval, yeast-like, single or budding cells measuring 1 to 3 μ in diameter. They appear to be surrounded by a clear halo with a dark central portion. These may be found free in the exudate, but generally are within the cytoplasm of macrophages and reticulo-endothelial cells (Fig. 13.8). In severe cases the cells may be packed with the organisms and they may even be found on smears of the peripheral blood. All materials should be submitted to the laboratory for culture and identification. This organism is fastidious in its growth requirements and often is difficult to culture. The laboratory personnel should be informed of the suspected diagnosis so proper steps may be taken and cultures held for a minimum of one month. Skin tests should be done with histoplasmin and due to cross immunity should include skin tests with blastomycin and coccidioidin.

Interpretation. A presumptive diagnosis of histoplasmosis may be made by the finding of intracellular yeast-like organisms in smears and wet preparations, but the definite diagnosis is dependent upon the isolation and identification of *Histoplasma capsulatum* by culture.

A skin test positive to histoplasmin is indicative of present or past

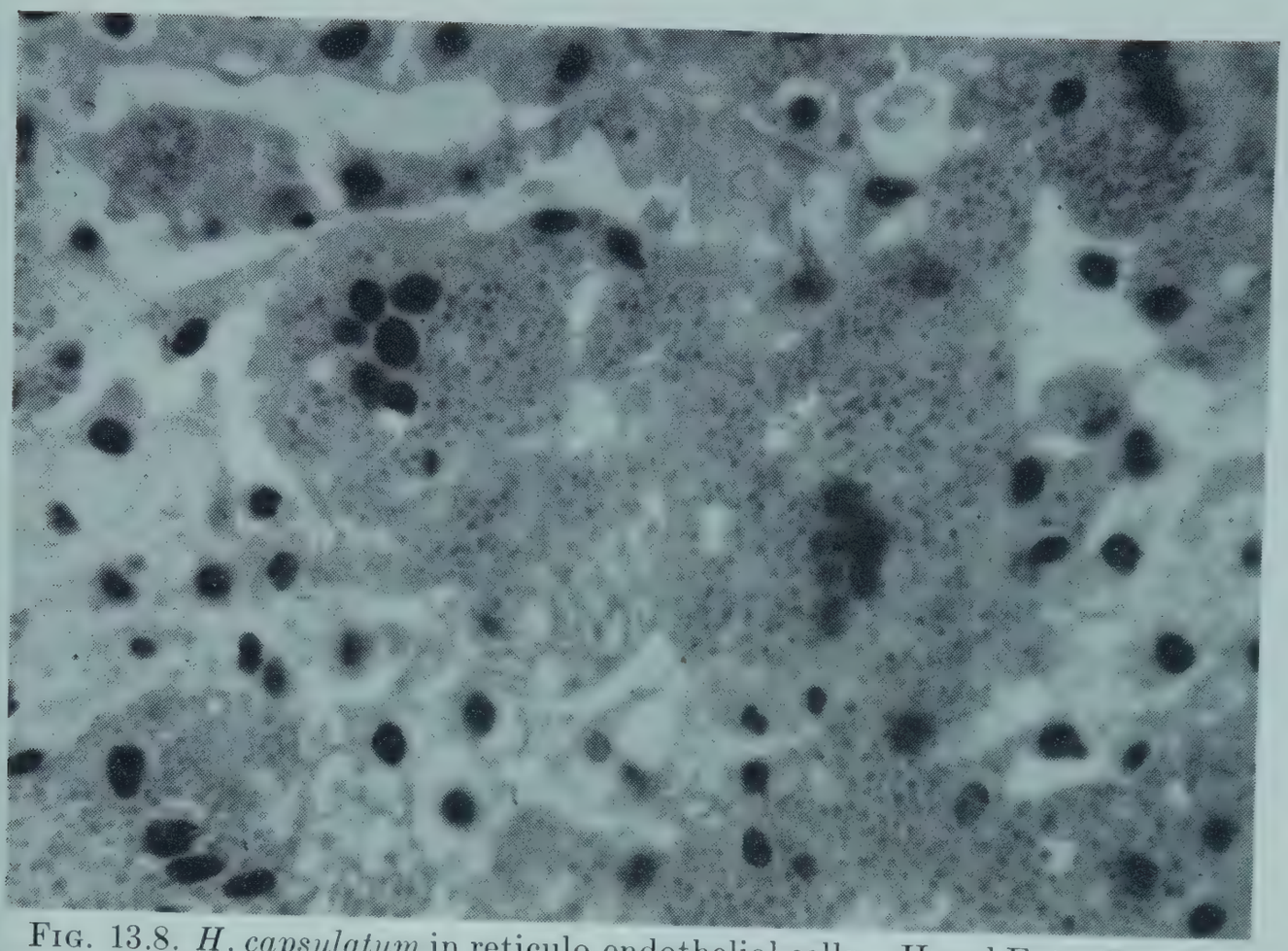


FIG. 13.8. *H. capsulatum* in reticulo-endothelial cells. H and E, × 430. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

infection and is interpreted similarly to the tuberculin skin test. A cross reaction to blastomycin occurs in approximately 50 per cent of patients and to a lesser extent with coccidioidin but the larger reaction to the histoplasmin skin test will allow separation of histoplasmosis cases. A test may become negative in the anergic stage of fulminating systemic infections. Complement fixation tests are made in some of the specialized laboratories. A high antibody response generally shows a good prognosis and a low antibody response a poor prognosis. Serial skin testing of a positive reactor will increase the antibody titer.

SPOROTRICHOSIS

Disease. Sporotrichosis is a chronic mycotic infection caused by *Sporotrichum schenckii*. It frequently begins as a small nodule at the site of inoculation. This breaks down, ulcerates and spreads to adjacent lymph nodes by way of the lymphatics, where it produces satellite indolent ulcers. The organism occurs in nature and patients generally give a history of contacts and injuries by plants or animals. The localized lymphatic form is the most common type of sporotrichosis. A systemic form may rarely be seen where no local lesion is noted with spread

to the lymphatics with or without pulmonary involvement. This systemic involvement of the lymph nodes does not produce ulceration unless the lesion is incised.

Diagnosis. This disease can not be diagnosed by scrapings and wet preparations. Fortunately the history, clinical signs and symptoms are frequently typical, and the awareness of the clinician of such a disease as sporotrichosis results in diagnosis. It should be considered in the differential diagnosis of any ulcerative lymphadenopathy. The specific diagnosis can not be made by biopsy as the lesions are non-specific granulomatous reactions and no organisms are demonstrated. It is by the culture of the scrapings, exudate, or aspirated material and recovery of *Sporotrichum schenckii* that a specific diagnosis of this disease is made Consequently, all materials must be submitted to the laboratory for culture and identification.

Interpretation. As this disease is diagnosed by the history, signs and symptoms it is imperative that the physician be alert for sporotrichosis. The specific diagnosis is made by a positive culture of *Sporotrichum schenckii* from material submitted from ulcers and lymph nodes.

COCCIDIOIDOMYCOSIS

Disease. Coccidioidomycosis is either an acute upper respiratory infection that is self limited, and involves the majority of persons living in endemic areas, or a chronic, progressive fulminating disease that involves practically every tissue in the body. It is caused by *Coccidioides immitis*. The endemic areas are the southwest region of the United States, including parts of Arizona, New Mexico, California and Texas. In the localized respiratory type of infection the individual has a non-specific upper respiratory infection of varying degree with a mild infiltrative pneumonitis. Approximately 7 per cent of infected individuals will develop an erythema nodosa type of hypersensitivity reaction within several days to weeks of the primary symptoms. Occasionally a primary ulcerative form may be seen. The systemic type of coccidioidomycosis will develop in less than 1 per cent of persons with the primary type and this predominates in males of the dark-skinned races. Dissemination occurs several weeks after the primary infection but may develop after several years. The symptoms will depend on the organs involved. Prognosis is poor.

Diagnosis. Suspected materials should be examined by wet preparation using a reduced light. Sputum, spinal fluid, pus from dermal abscesses and exudates from ulcers may be cleared by use of 10 per cent

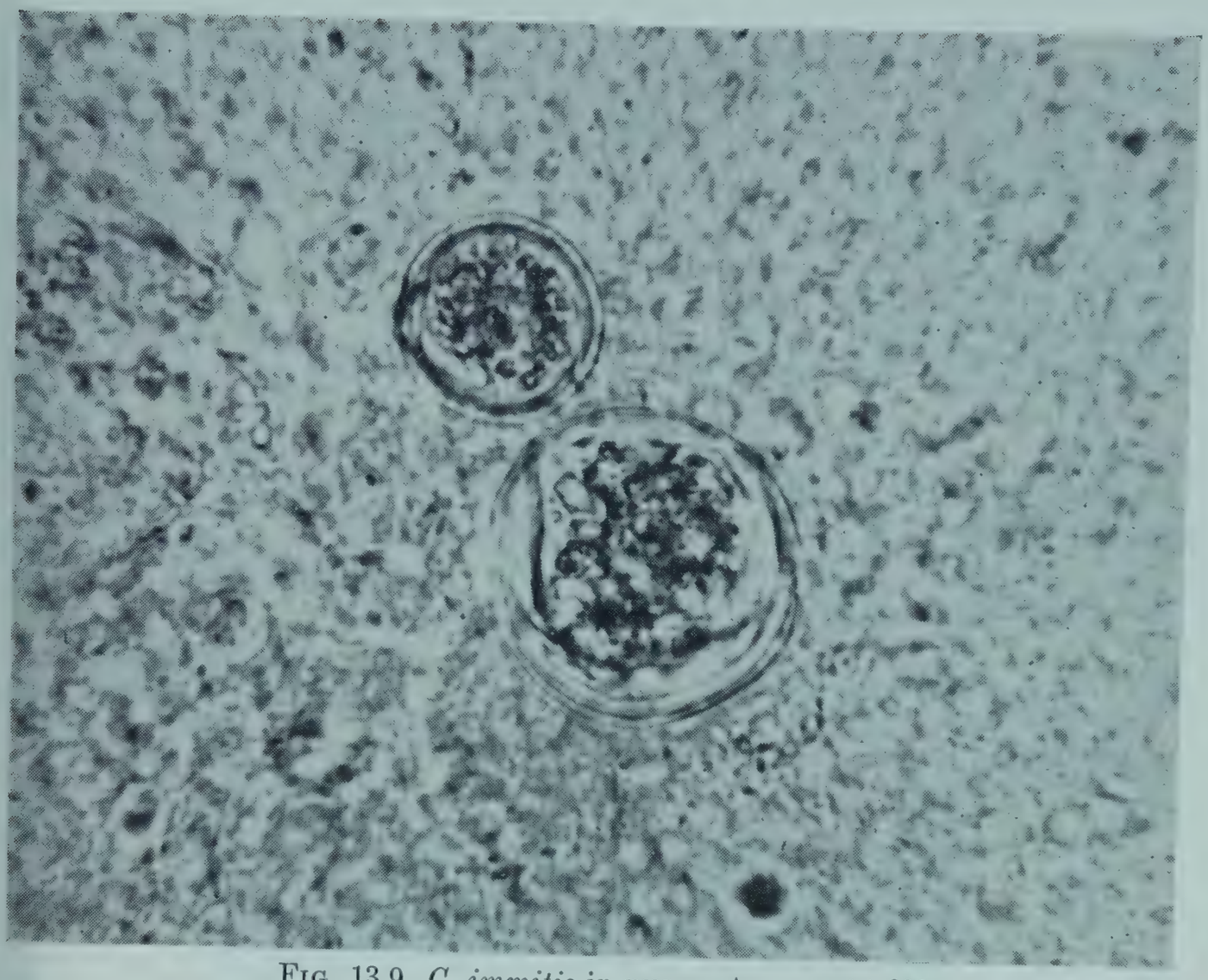


FIG. 13.9. *C. immitis* in pus, wet prep., × 600

KOH if necessary and carefully searched for the diagnostic thick-walled spherules that measure between 10 and 70 μ in diameter. These spherules are non-budding and the larger are filled with numerous small endospores that measure 2 to 5 μ in diameter (fig. 13.9). If the spherule has ruptured, the shell may be seen and the released endospores will be similar to the non-budding cells seen in exudates in blastomycosis. Skin tests should be made using coccidioidin, histoplasmin and blastomycin. Laboratories in endemic areas may do precipitin and complement fixation tests on blood serums submitted to them.

Interpretation. The history of a person living in or visiting an endemic area should suggest the possibility of coccidioidomycosis in any patient with upper respiratory tract infection, or presenting erythema nodosa or erythema multiforme lesions, or in any chronic disease characterized by ulcerations, dermal abscesses or visceral changes. The finding of the large endospore filled spherules is common, but in many cases these may not be found, and the diagnosis is made by positive culture of *Coccidioides immitis* from exudates, tissues or sputum submitted

to the diagnostic laboratory. The skin test becomes positive approximately a month after exposure or a week after symptoms develop, and may be very strong in the primary disease associated with manifestations of hypersensitivity. The precipitin test is positive in recent disease but reverts to negative within several months. The complement fixation test will show titers after several months and a continued rise of these with a poor skin test reaction, or a skin test that decreases in intensity when repeated, indicates spread to the systemic form.

CHROMOMYCOSIS

Disease. Chromomycosis is a chronic granulomatous infection of the dermis produced by any one of numerous organisms belonging to the *Hormodendrum*, *Phialophora* or *Cladosporium* family. The majority of cases are produced by *Hormodendrum pedrosoi*, *Hormodendrum compactum* or *Phialophora verrucosa*. Usually the lesions are found on the extremities or exposed parts of manual laborers. The lesion begins as a papule which slowly spreads and produces a nodular, verrucous, firm,

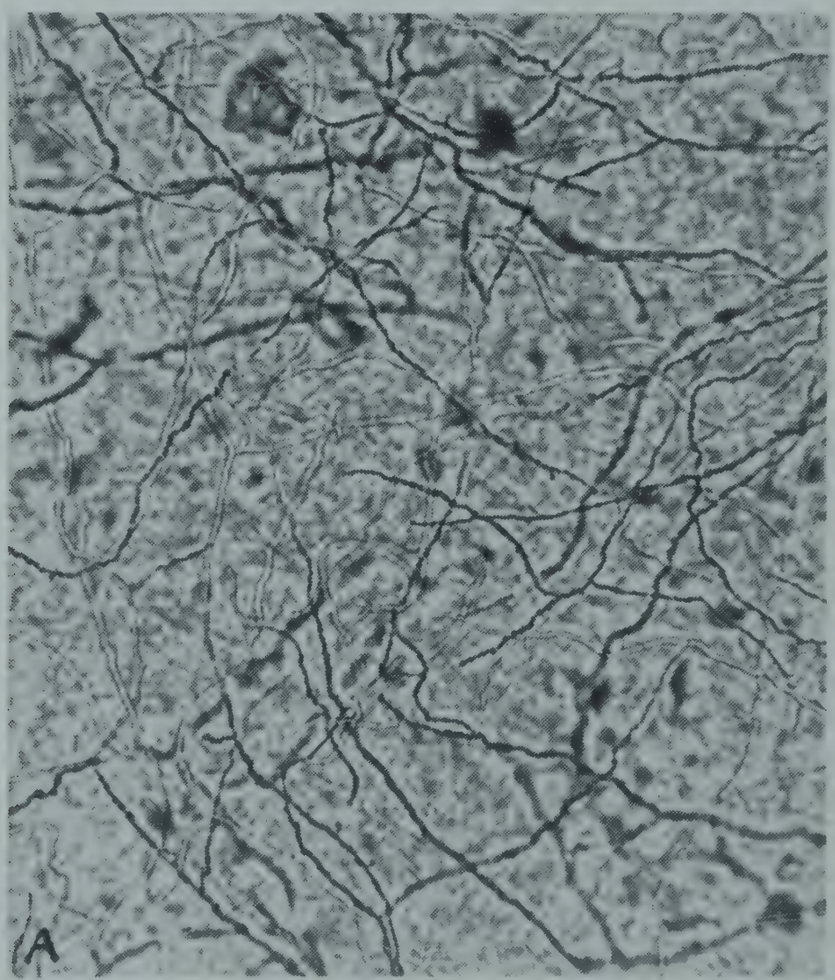


FIG. 13.10. Skin with septate hyphae, KOH preparation, × 150. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

purplish tumor. Spread may be extensive or the lesion may be solitary. Ulceration is rare unless secondary infection occurs.

Diagnosis. Scrapings or exudate or even a portion of the lesion should be removed, cleared in KOH (10 per cent) and examined for the presence of single or multiple, dark brown, thick-walled organisms that measure from 6 to 10 μ in diameter. The cells do not multiply by budding but divide by fission. The species can not be determined from the appearance of the organism found in the lesion, but must be cultured by the laboratory and the fungus identified. Biopsies usually are positive. Occasionally only irregular, broad, septate branching hyphae are present. These organisms usually belong to species of the genera *Hormodendrum*, (*Cladosporium*) which are often considered nonpathogenic. They are capable, however, of producing fatal brain abscesses or localized granulomas.

Interpretation. A verrucous lesion found on the exposed portion of the extremity, especially with a history of trauma, should be examined microscopically and cultured. In a wet preparation the presence of

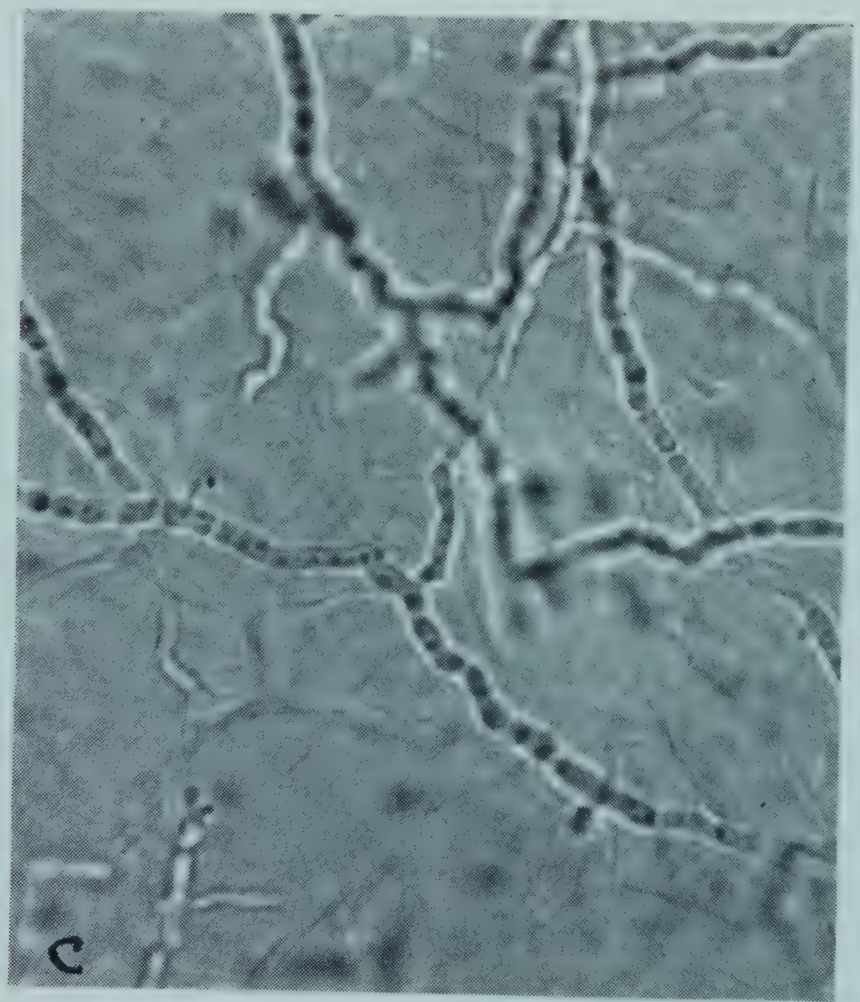


FIG. 13.11. Skin with septate hyphae, KOH preparation, × 500. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

dark brown, thick-walled organisms dividing by fission is presumptive evidence of chromomycosis. The material must be cultured and the specific etiologic agent identified.

DERMATOMYCOSIS

Disease. Dermatomycoses are fungous infections of the skin and its appendages produced by species of the genera *Trichophyton*, *Microsporum*, *Epidermophyton* and occasionally *Candida*. Clinical classification of the disease is based on the clinical manifestations and area of the body involved, since various species of the different genera produce similar diseases on various parts of the body. The most common names used are tinea capitis (head), tinea barbae (chin), tinea corporis (body) tinea cruris (genital area), tinea unguium (nails), and dermatophytosis of hands and feet (tinea pedis). These diseases produce very little inflammatory reaction of the dermis, but may be macular or papular and are often associated with itching. Hair follicles and even hairs may be invaded by some species of fungi. Dermatophytid (id) reac-



FIG. 13.12. Trichomycosis axillaris, hairs with surrounding sheath of organisms, × 100. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

tions may occur with these infections, and the itching vesicles should not be confused as being the site of infection which is often elsewhere on the body. Suppurative infections of the dermis generally are the results of secondary bacterial invasion superimposed on an underlying fungus infection that has been macerated or irritated by scratching or by over vigorous treatment.

Diagnosis. Scrapings of the infected skin, nails, or hairs should be cleared by gently heating the material in 10 per cent KOH and examined for the presence of branching hyphae that may be segmented. It is impossible to separate the species by observation of the wet preparation, but *Trichophyton* species involve skin, hair and nails, *Microsporum* species skin and nails. The material should also be cultured for specific identification of the etiologic agent, as this is the only positive method of identification.

Interpretation. The finding of mycelial elements in skin, hair or nails is the simplest method of diagnosing these fungous diseases, but additional cases may be diagnosed by culture as well as the etiologic agent identified in cases known to be fungal in origin (Figs. 13.10 and 13.11). Cultures should be done routinely as the prognosis of the case depends

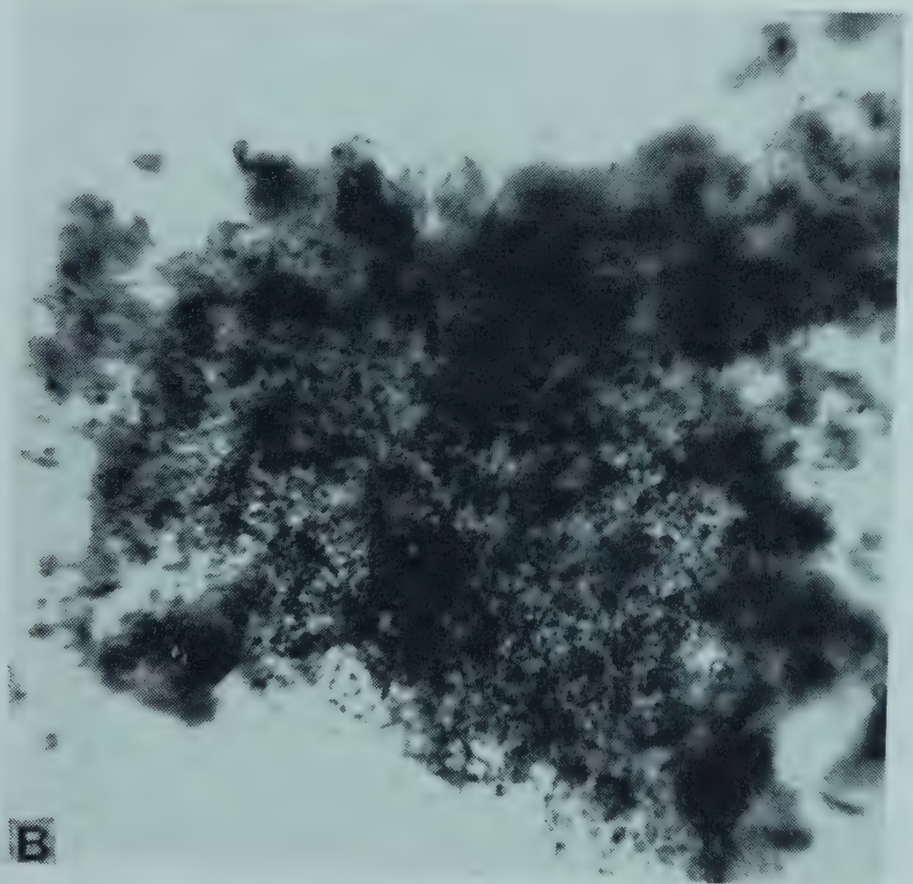


FIG. 13.13. Erythrasma, branching nocardial filaments, × 430. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

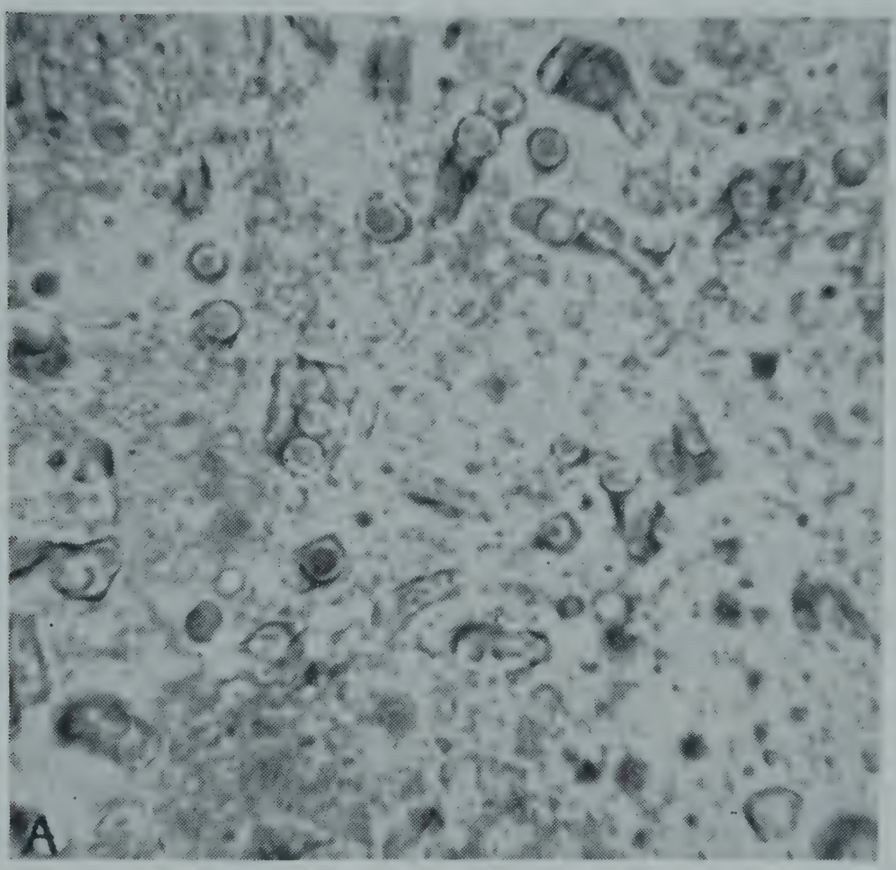


FIG. 13.14. Tinea versicolor, wet prep., × 700. From Moss and McQuown Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.



FIG. 13.15. Tinea versicolor, wet prep., × 850. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

on the fungus identified, and the method of treatment depends to a large extent on the species present.

TRICHOMYCOSIS AXILLARIS

Trichomycosis axillaris is produced by infection with *Nocardia tenuis* and involves the hairs of the axilla and pubic region. The hair shafts are encircled or partially encircled by nodules that may be white, yellow or red. If the area is moist the nodules are soft. The clothes may be stained. The disease is diagnosed by the presence of these nodules on the hairs (Fig. 13.12). When crushed the nodules are composed of innumerable intertwining, branching hyphae, measuring less than 1 μ in diameter, which tend to fragment and produce bacillary forms. Cultures generally are not necessary.



FIG. 13.16. Piedra, nodule on hair shaft, × 125. From Moss and McQuown: Atlas of Medical Mycology, The Williams & Wilkins Co., Baltimore, 1953.

ERYTHRASMA

Erythrasma is a chronic superficial infection of the axillae and inguinal areas produced by *Nocardia minutissima*. It generally produces no symptoms. The lesions are macular, brownish tan, and are covered with fine bran-like scales. Diagnosis is made by the typical clinical picture. Scales cleared and examined microscopically show intertwining mycelial elements measuring less than 1 μ in diamter which fragment and may appear like bacilli (Fig. 13.13).

TINEA VERSICOLOR

Tinea versicolor, produced by *Malassezia furfur*, results in chronic, asymptomatic or slightly pruritic tan macules that are found over the chest, back, shoulders and neck. In the summer time the macules may appear depigmented as the normal skin tans more readily. The diagnosis is made by the clinical picture. Desquamated material if cleared and examined microscopically will contain short, curved or angular fragments of mycelium surrounding groups of round, budding thick-walled spores, measuring 3 to 5 μ in diamter (Figs. 13.14 and 13.15). The organisms have not been grown on artificial culture media.

PIEDRA

Piedra is characterized by hard white or black nodules located along the shafts of hairs. It is produced by either *Piedraia hortai* or *Trichosporon beigelli*. No symptoms are produced but the hairs are irregular and gritty (Fig. 13.16). When hairs and concretions are cleared, crushed and examined microscopically, they show branching hyphae that tend to break up into rectangular arthrospores. The hair is not involved. Cultures are not necessary.

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CHAPTER 14

MEDICAL PARASITOLOGY

By Seward E. Miller

In the past, many physicians and medical students in the United States were apt to think that significant animal parasitic diseases were mostly confined to the tropics and in fact constituted the major distinguishing characteristic of tropical medicine. While it is quite true that some tropical and subtropical environments are particularly conducive to large numbers of parasitic infections, still today, more than ever, no physician can afford to ignore these parasitic infections, for many are common in the United States and, with the increasing travel and extensive temporary residence abroad of large numbers of our armed forces and nonmilitary citizens, practically all the human parasitic infections are being seen in our hospitals and clinics. Moreover, the increased attention given to the diagnosis of our native parasitic diseases in recent years has demonstrated their widespread prevalence throughout the United States, particularly *Endamoeba histolytica* infection.

Explosive epidemics of parasitic infections are much less common than bacterial. Parasitic infections are more prone to be insidious in onset, protean in symptomatology, and chronic in character. However, to prevent ultimate serious destructive pathology, physicians and laboratories must constantly be alert to detect the early stages of these infections, for excellent chemotherapeutic measures exist for many of these diseases.

Some parasites appear to produce no symptoms, and even when present in large numbers are incapable of producing any pathologic alteration in the human host. This further enhances the laboratory's responsibilities in accurately identifying all animal parasites. As might be expected with such insidious chronic infections, only a few produce in the host sufficient antibody reaction for skin and serological diagnostic techniques to be employed successfully. Therefore, in general, their detection depends ultimately upon the morphological identification of these parasites.

In order to view these parasites in proper perspective, it is well to recall the standard classification of all living organisms—plant and animal. The medically important diseases of plant origin are the bacterial, and those due to the higher, more complex forms of plant life, the mycological. These are discussed in Chapters 12 and 13. The

animal parasites infecting man will be examined in this chapter. Here, indeed, exist some of the most intriguing and at times complex host-parasite relationships known.

Following the International Code of Zoological Nomenclature, all parasites belong to a phylum, class, order, family, genus, and, finally, species; occasionally subspecies are designated. In reverse, starting with the grouping of all individuals who appear alike into one species:

Species—individuals, alike in all respects
Genus—species, alike in main characteristics
Family—genera, with close resemblances
Order—families, agreeing in some striking characteristic
Class—orders, agreeing in specific characteristics
Phylum—classes, similar in general character

Again, animal parasites can be divided conveniently into two groups—the unicellular protozoa and the multicellular metazoa.

The term *parasite* is applied to the animal that obtains food and shelter from the host and in short derives most of the benefit from the association. The host may suffer a great variety of ill effects and ultimately perhaps even death. Again it should be emphasized that many human parasitic infections are apparently non-pathogenic. Parasites which live on the exterior surface of the body are termed ectoparasites and these conditions are termed *infestations*. Parasites which inhabit the interior of the body are termed endoparasites and these conditions are called *infections*. Thus, skin and hair animal parasitic diseases are *infestations* and all internal animal parasitic diseases, *infections*.

The terminology by which specific parasites are designated is composed of two names—first the genus, in Latin or Greek, which always is capitalized, followed by the species name which is not capitalized (note examples in Table 14.1). Frequently, the genus name is abbreviated to its first capital letter; thus, *E. histolytica* is quite commonly used.

THE PROTOZOA

Table 14.1 shows the phylum Protozoa divided into four classes: the Sarcodina, Infusoria, Mastigophora, and Sporozoa, with their chief distinguishing characteristic. The Protozoa are all complete single-celled animals with no differentiation into tissues.

INTESTINAL AND GENITAL AMEBAE

General Considerations

The six intestinal amebae of man occur in a varying per cent of the population throughout the temperate and tropical zones. Their distri-

TABLE 14.1

Medically Important Animal Parasites

Phylum	Class	Distinguishing Characteristic	Example
PROTOZOA (single cell)	Sarcodina	Pseudopodia for locomotion	*Endamoeba histolytica*
	Infusoria	Cilia for locomotion	*Balantidium coli*
	Mastigophora	Flagella or undulating membrane for locomotion	*Trichomonas vaginalis*
	Sporozoa	No locomotion—form spores	*Plasmodium vivax*
PLATYHELMINTHES (flat worms)	Cestoda	Ribbon or tapeworms	*Taenia saginata*
	Trematoda	Oval or leaf-shaped (flukes)	*Schistosoma hematobium*
NEMATHELMINTHES (round worms)	Nematoda	Spindle-shaped, tapering at both ends	*Trichuris trichiura*
ARTHROPODA	Insecta	Six-legged insects	*Pediculus pubis*
	Arachnida	Four pairs of legs	*Saroptes scabei*

bution is fairly uniform throughout the United States, with the greatest incidence occurring usually among institutionalized groups. *Endamoeba coli* is the commonest ameba, being present in over 30 per cent of the population. The incidence of *Endamoeba histolytica* and *Endolimax nana* is somewhat less than half as great, or about 13 to 14 per cent, while *Dientamoeba fragilis* and *Iodamoeba bütschlii* are found in only 4 per cent of the population. *Endamoeba gingivalis* is found in 10 to 20 per cent of healthy mouths and in a high percentage of patients with pyrrohea alveolaris and those with receding spongy gums.

Poor sanitation and unhygienic habits enhance the spread of these amebic infections and their control depends upon clean environmental sanitation and individual hygiene.

The term amebiasis denotes an infection with *Endamoeba histolytica*, the only pathologically destructive amebae. Of the other five amebae, four are nonpathogenic. The fifth, *Dientamoeba fragilis*, causes only a mild colitis in some people. Actually, therefore, these latter five amebae are of medical importance chiefly because they must be distinguished from *E. histolytica* in order to avoid erroneous diagnosis.

There are seven flagellates found in the intestinal and genital tracts.

TABLE 14.2

Protozoa Inhabiting Intestinal and Genital Tracts

Name, Genus and Species	Disease	Habitat
Sarcodina (amebae)		
Endamoeba histolytica	Amebiasis	Colon
Endamoeba coli	Nonpathogenic	Colon
Endamoeba gingivalis	Nonpathogenic	Mouth
Endolimax nana	Nonpathogenic	Colon
Iodamoeba bütschlii	Nonpathogenic	Colon
Dientamoeba fragilis	Colitis—mild	Colon
Mastigophora (flagellates)		
Trichomonas hominis	Nonpathogenic	Colon
Trichomonas vaginalis	Vaginitis; urethritis-prostatitis	Vagina; urethra-prostate
Trichomonas tenax	Nonpathogenic	Mouth
Chilomastix mesnili	Nonpathogenic	Colon
Embadomonas intestinalis	Nonpathogenic	Intestines
Enteromonas hominis	Nonpathogenic	Intestines
Giardia lamblia	Giardiasis or lambliasis	Duodenum
Infusoria (ciliate)		
Balantidium coli	Balantidial dysentery	Colon

Of these, *Trichomonas vaginalis* is pathogenic in the genital tract and *Giardia lamblia* in the intestinal tract. In addition, there is one ciliate, *Balantidium coli*, which may cause a serious ulcerative intestinal disease. Two of these infections, the amebic *Endamoeba histolytica* and the ciliate *Balantidium coli*, can be most serious. The two pathogenic flagellate infections, *Trichomonas vaginalis* and *Giardia lamblia*, cause varying annoying and irritative symptoms without greatly destructive lesions. The other protozoal infections are of little or no significance except as they might be mistakenly identified as a pathogenic protozoa.

Although there is considerable controversy over the habitat of these intestinal protozoa, mostly they appear to inhabit the colon, except Giardia, which inhabits the duodenum and jejunum.

Most of the intestinal protozoa exist in two distinct forms—a motile form, or *trophozoite*, and a non-motile form, *cyst*. The motile trophozoites are the active, feeding forms and are usually so fragile that they die and disintegrate rapidly upon leaving the host. The cysts, on the

other hand, are resistant to cooling and to some degree of drying and remain recognizable in the stools for several days after they are passed. Encystment occurs in the intestine as the protozoa pass downward from their usual place of residence. If the intestinal contents move rapidly there is not sufficient time for cysts to develop and only motile forms will be found in the stools. This occurs if diarrhea is present or if a purge has been given. In mushy stools both motile forms and cysts may be found; in formed stools only cysts are usually found. Cases of amebic dysentery may have ulcers at any level from the cecum (rarely the terminal ileum) to the rectum, and these ulcers vary greatly in acuteness, extent, and severity. If they are present only in the upper part of the colon and if diarrhea is not present, only cysts are likely to be found in the stools. If the ulcers are only in the sigmoid or rectum, only motile forms are likely to be found. Only one of the intestinal amebae does not form cysts (*Dientamoeba fragilis*). Cysts usually are easier to identify than motile forms.

It is important to supplement examination of routine stool specimens by examination of a purged specimen (unless diarrhea is already present), if a complete study of the protozoal fauna is to be made. If a purge is given, the second evacuation is the best one for the detection of motile forms. The first evacuation should be examined also, but it may contain only material from the rectum.

Not every stool specimen will reveal protozoa even if they are present in the intestinal tract, for much depends upon their number and upon cycles in their multiplication. In general, only about 50 per cent of the persons actually harboring any one species of protozoa can be detected by one examination of a formed stool, and about 75 per cent by one examination of a liquid stool. It usually requires six examinations of formed stools obtained on different days, or three examinations of liquid stools or stools following purges, obtained on different days, to detect practically all infected persons.

In acute amebic dysentery, motile amebae containing red blood cells can usually be found easily in the stool specimens. Small particles of mucus mixed with blood furnish the best material. In subacute or chronic cases it may be necessary to examine material direct from ulcers by means of a proctoscopic or sigmoidoscopic examination.

Collection of Specimens

In order to find characteristic motile forms the stool specimen must be examined as soon as possible after being passed. Specimens more

than one or two hours old are of little or no value. A bed-pan specimen is best because it affords opportunity to see the whole specimen grossly and to select the most suitable portion for examination. It is not advisable to keep a specimen warm because the amebae degenerate rapidly under such conditions. It is usually unnecessary to use a warm stage in examining for motile forms. Although motility may be suspended temporarily in a slide preparation, it is usually resumed after a few minutes. If suspicious non-motile forms are found the slide may be warmed to body temperature on the microscope lamp.

E. histolytica may be demonstrated in the chocolate-colored purulent material from amebic abscesses of the liver or other parts of the body or from beneath the overhanging edges of amebic ulcers of the skin. If the amebae are not found in the pus from an abscess cavity, they may be present in scrapings from the wall of the abscess or in the drainage fluid a day or two after operation. Great care must be taken to differentiate them by their characteristic active motility from body cells.

Specimens obtained by proctoscopic or sigmoidoscopic examination should receive special attention. A person qualified to identify *E. histolytica* should be present at the examination, equipped with a microscope, slides, coverslips, normal saline and toothpicks. Specimens should be obtained directly from lesions either by a blunt curette or by a long glass tube bent and drawn to a small smooth opening at one end and fitted to a strong 5-ml. rubber bulb for suction at the other end. Cotton swabs should be avoided except for cleansing the area, because the material is absorbed into the cotton and may be lost. Specimens obtained by the curette or glass tube should be examined at once in slide coverslip preparations, using saline only if too thick for mounting by simple pressure between slide and coverslip.

Examinations for cysts may be made at any time up to 24 hours if the specimen has been kept at room temperature or up to several days if it has been refrigerated.

The rapid disintegration of amebic trophozoites, which may be the only forms present in the feces of acute cases, has been a tremendous handicap in the laboratory dignosis of amebiasis. Brooke and Goldman have devised a novel technique for preserving these fragile trophozoites so that the material may be examined hours, days, or even weeks later with the trophozoites remaining intact. Polyvinyl alcohol, a synthetic plastic material dissolved in Schaudinn's fixative solution, may be used as a preservative in two ways. A smear may be prepared on a glass slide by mixing a drop of fecal material with 3 drops of the P.V.A.

fixative and spreading it evenly over about one-third of the glass slide; to insure thorough drying of the smears it is well to place them in a 37°C.-incubator overnight. The second method is to mix a small portion of the fecal material in a small vial containing about 3 parts P.V.A. fixative. Smears for staining may be prepared at once or any time, even months later. The smears or the vial of P.V.A. and fecal material should be sent to a laboratory employing an iron-hematoxylin stain so that critically stained organisms may be observed for diagnosis.

Examination for Motile Protozoa

Place a drop of normal saline on a glass slide, mix a small portion of the specimen with the saline and cover with a coverglass. The emulsion should be thin enough so that ordinary print easily may be read through it. In cases of dysentery, dilution of the material may not be necessary. Samples should be taken from various parts of the specimen. Not less than three preparations should be examined from each specimen. It should be remembered that several species of protozoa may be present. Examine the entire preparation under the low power of the microscope, using the high dry magnification and the oil-immersion for identification of suspicious objects.

Examination for Cysts

Most cysts can be identified in saline preparations; however, for clearer details of morphology and nuclei, Lugol's iodine solution is most useful.

Place a small drop of Lugol's solution on a slide and emulsify thoroughly a very small particle of fecal material. The emulsion again should be of such a density that newspaper print easily can be read through it. Cover at once with a coverglass and examine the whole coverglass area under low power of the microscope. Identify suspicious objects with the high dry or oil-immersion lens. Examine a portion of the area with high dry magnification for the smaller cysts. Cysts may appear green, yellow, or orange; glycogen vacuoles appear brown.

Iron Hematoxylin Stain for Intestinal Protozoa

It is sometimes difficult, especially for an inexperienced observer, to be sure of the identification of an intestinal protozoon in the saline iodine preparations. For this reason every laboratory should be prepared to make fecal smears stained with iron hematoxylin preparations. Such stained preparations bring out details of morphological structure beau-

tifully and thus greatly enhance and facilitate the identification. The identification also is more reliable. Formed or mushy stools or those containing mucus make better fixed preparations than fluid stools, which are likely to wash off the slide during the procedure of fixation. Figs. 14.1 and 14.2 show the characteristics of the intestinal protozoa stained by this method.

Concentration of Cysts. Zinc Sulfate Centrifugal Flotation

(1) Emulsify about 1 gm. of feces in 10 ml. of tap water. Strain through one layer of wet cheesecloth in a small funnel into a Wassermann tube.

(2) Centrifugalize at 2500 r.p.m. for 1 minute. Pour off supernatant fluid, add about 3 ml. of water, suspend sediment and fill tube with water.

(3) Repeat (2) 3 or 4 times until supernatant fluid is clear. Pour off last supernatant.

(4) Add about 3 ml. zinc sulfate solution sp. gr. 1.180 (33 per cent solution), break up sediment and add zinc sulfate solution to one-half inch of the rim.

(5) Centrifugalize at 2500 r.p.m. for 1 minute.

(6) Using a wire loop of about 5 mm. diameter, remove several loopsful of surface film to a glass slide.

(7) Mix with one drop of iodine solution, mount with a coverslip and examine under low and high magnification.

Cysts of protozoa and eggs of hookworm, Trichocephalus, Ascaris and Enterobius are concentrated and floated by this method. Amebic cysts may show shrinkage away from wall but are still satisfactory for identification.

Identification of Intestinal Protozoa

Amebae must be differentiated first of all from large mononuclear phagocytes and epithelial cells of the intestine. Epithelial cells are never motile and have clear cytoplasm and large nuclei. Large mononuclear phagocytes may be slightly motile and may contain red blood cells, leukocytes or pink-colored vacuoles. They are often mistaken for *Endamoeba histolytica* but can be differentiated by having a much more delicate cell membrane and a larger, oval, prominent nucleus. All of the amebae have a highly refractile outline.

The important point is to differentiate *E. histolytica* from the other amebae (Figs. 14.1 and 14.2). The safest rule to follow is to identify a motile ameba as *E. histolytica* only if it contains red blood cells. The second important differential feature is the type of motility. *E. histolytica*, when normally active, moves rapidly in a single direction with a streaming movement (Fig. 14.3), whereas the other amebae throw out pseudopods in one direction or another without progressing to any great

TABLE 14.3

Differential Features of Motile Amebae in Fresh Preparations

	Size	Pseudopodia	Progressive Movement	Endoplasm	Vacuoles	Food Inclusions	Nucleus
	μ						
Endamoeba histolytica	15–30 (7–40)	Hyaline	More rapid	Finely granular	None or few	Usually absent, except red blood cells	Inconspicuous
Endamoeba coli	18–40	Granular	Sluggish	Coarsely granular	Few or many	Numerous, bacteria and fecal debris. No red blood cells	Conspicuous
Endolimax nana	6–12	Hyaline, very blunt	Sluggish	Finely granular	Few or many	Numerous, bacteria, etc.	Inconspicuous
Iodamoeba bütschlii	9–14 (5–20)	Granular	Sluggish	Coarsely granular	Many	Numerous, bacteria, etc.	Inconsipcuous
Dientamoeba fragilis	3.5–12	Hyaline	Rapid pseudopods, little progress	Finely granular	Few or many	Bacteria, etc.	Usually two, very indistinct

distance. When less active, *E. histolytica* may simulate the movements of the other amebae, but its pseudopods are usually larger, are definitely hyaline in appearance, and if watched long enough the endoplasm usually flows into the pseudopod causing the ameba to change its position. Unless it is undergoing degeneration *E. histolytica* usually contains no vacuoles, whereas the other amebae are usually highly vacuolated, the vacuoles often containing bacteria. Occasionally such vacuoles may have a pink color and must not be mistaken for red blood cells, which have a rusty yellow color. The nucleus of *E. histolytica* is almost invisible in unstained preparation, while that of *E. coli* is distinct. The nuclei of the other amebae are very indistinct. In size *E. histolytica* varies greatly but is usually smaller than *E. coli* and larger than the other amebae. Dientamoeba is difficult to differentiate from Endolimax in fresh preparations, although when actively motile it can be distinguished by its film-like pseudopodia with sharp points. The final differentiation should be made with the iron hematoxylin stain.

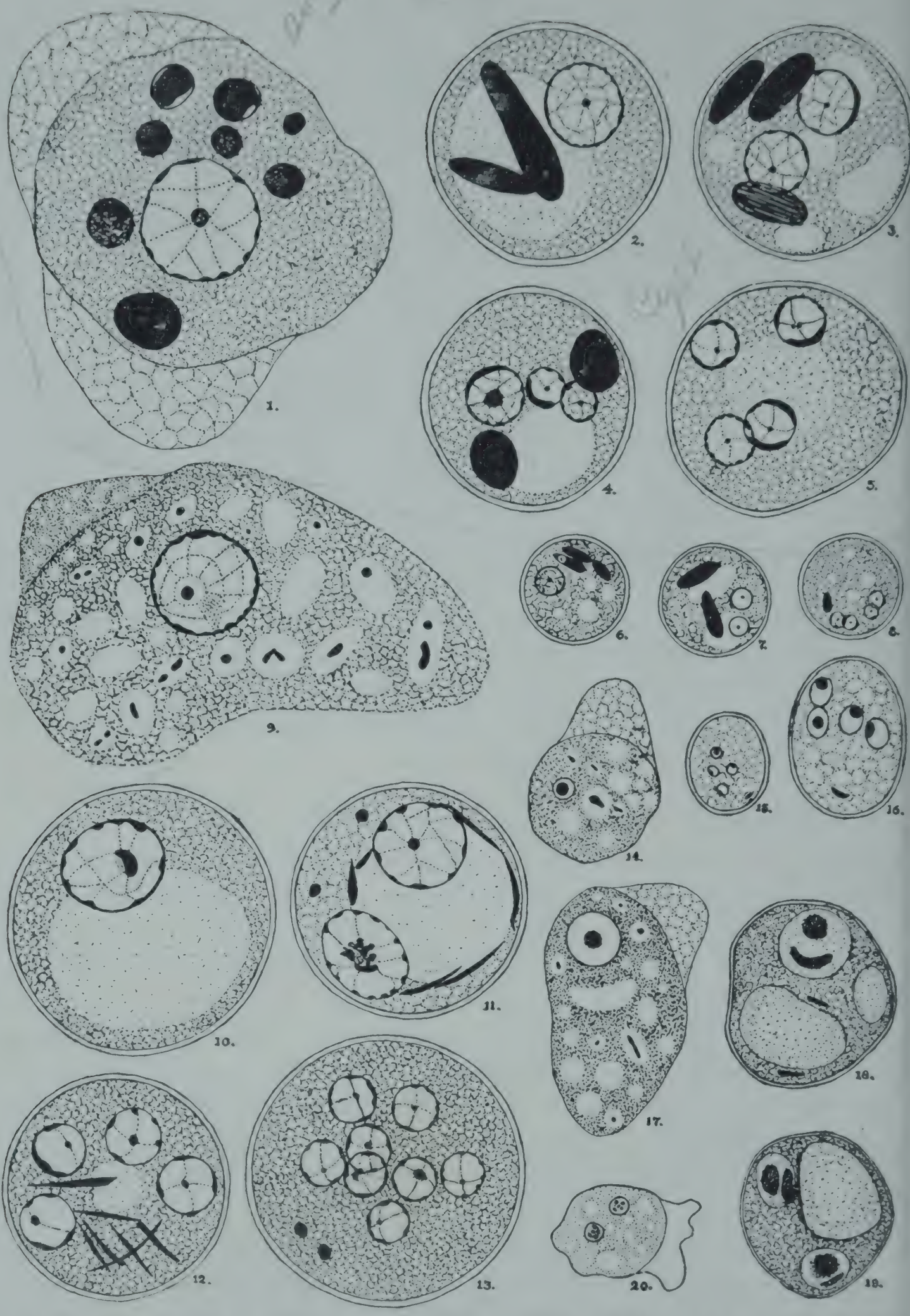
Table 14.3 gives the principal differential features of the intestinal amebae of man in unstained preparations.

Cysts as Seen in the Iodine Preparations (Fig. 14.4)

In the process of encystment amebae become spherical, the cell membrane becomes thickened, a characteristic number of nuclei is developed, and glycogen vacuoles as well as "chromatoidal bodies" are sometimes formed. The following characteristics of amebic cysts can be distinguished with iodine.

The cysts of *Endomoeba histolytica* (Plate 14.4 [figs. 12–15]) vary in size from 6 to 15 micra, from the small to the large race. The larger cysts may be confused with those of *Endomoeba coli*, the smaller ones with *Endolimax nana*. The cyst is usually spherical but may be oval. When fully developed it has four nuclei, but immature cysts with one, two or three nuclei are often found. The nuclei are spherical and have a distinct rim and a central dot, the karyosome, both of which are refractile with iodine. The cytoplasm is glassy clear. In fresh specimens there may be a large or small brown-staining goycogen vacuole, indefinite in outline, and usually found only in one- and two-nucleate cysts. Chromatoidal bodies should be sought only in saline preparations since they are usually invisible in iodine.

The cyst of *E. coli* (Fig. 14.4 [figs. 16–18]) is usually larger than that of *E. histolytica*, and has 1 to 8 nuclei. The nuclei are more heavily stained than those of *E. histolytica* and the karyosome is usually ec-



FIGS. 14.1 AND 14.2. Intestinal protozoa. Figures 1 to 19, 21 to 36, from camera lucida drawings by Dr. John F. Kessel, originally published to illustrate "The Distinguishing Characteristics of the Intestinal Protozoa of Man," China Medical Journal, February 1925. Figure 20, after Thompson and Robertson, "Protozoology," page 141. Figures 1 to 34 × 2000. Figures 35 and 36, × 650.

Fig. 1. *Endamoeba histolytica*, motile form containing red blood cells.
Figs. 2 5. *E. histolytica*, large race, one-, two-, three-, and four-nucleated cysts.
Figs. 6–8. *E. histolytica* cysts, small race.
Fig. 9. *Endamoeba coli*, motile form.
Figs. 10–13. *E. coli*, one-, two-, four-, and eight-nucleated cysts.
Fig. 14. *Endolimax nana*, motile form.
Figs. 15–16. *E. nana* cysts.
Fig. 17. *Iodamoeba bütschlii*, motile form.
Figs. 18–19. *I. bütschlii*, one- and two-nucleated cysts.
Fig. 20. *Dientamoeba fragilis*, motile form.

FIG. 14.2



Fig. 21. *Chilomastix mesnili*, motile form.
Figs. 22–23. *C. mesnili* cysts.
Fig. 24. *Giardia lamblia*, motile form, ventral view.
Fig. 25. *G. lamblia*, motile form, lateral view.
Figs. 26–27. *G. lamblia* cysts.
Fig. 28. *Trichomonas hominis*, motile form.
Fig. 29. *T. hominis*, non-motile form (cyst?).
Fig. 30. *Balantidium coli*, motile form.
Fig. 31. *Blastocystis hominis* (a yeast, non-pathogenic).
Figs. 32–34. Yeast.
Fig. 35. Cellular exudate, amoebic dysentery, showing motile amoebae, a large mononuclear leucocyte, red blood cells, and Charcot-Leyden crystals.
Fig. 36. Cellular exudate, bacillary dysentery, showing polymorphonuclear leucocytes, large mononuclear leucocytes, and red blood cells. Two large mononuclear leucocytes contain red blood cells, another without the nucleus contains black staining vacuoles.


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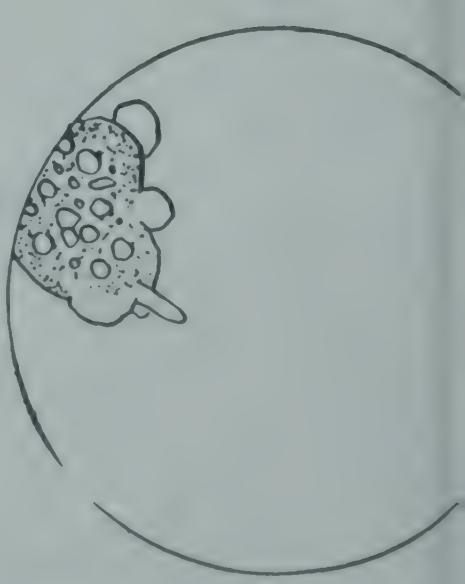

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FIG. 14.3. Showing the various changes in shape and position of a single vegetative motile *Endamoeba histolytica*, the changes taking place at intervals of a few seconds.

centrically placed. The cytoplasm is evenly granular, often like a fine network. The glycogen vacuole, if present, is usually similar to that of *E. histolytica*. Since glycogen is used as food it is not found in older cysts. In 2-nucleate cysts, however, the glycogen vacuole may occupy nearly the entire cyst and may press the nuclei against the cyst wall. Chromatoidal bodies should be sought in saline preparations since they are not visible with iodine.

The cyst of *E. nana* (Fig. 14.4 [figs. 19–20]) is usually smaller than that of *E. histolytica* and is generally oval in shape. The cyst wall is thin. There are 1 to 4 nuclei, but they are difficult to see. They are very small and appear as refractile granules. The cytoplasm is finely granular and more hyaline in appearance than that of the other amebae. There are usually no glycogen vacuoles or chromatoidal bodies.

The cyst of Iodamoeba (Fig. 14.4 [fig. 21]) varies in size from that of *E. nana* to that of a medium sized *E. histolytica*. It varies greatly in shape, being round, oval or irregular. Its most prominent and constant feature is a large dark brown glycogen vacuole having a very distinct outline. However, older cysts that have consumed their glycogen can be confused with *E. histolytica* cysts. There is one nucleus (rarely two), displaced to one side, fairly distinct in outline, with no visible karyosome.

Dientamoeba fragilis does not possess a cyst form.

Table 14.4 summarizes the differential features of amebic cysts.

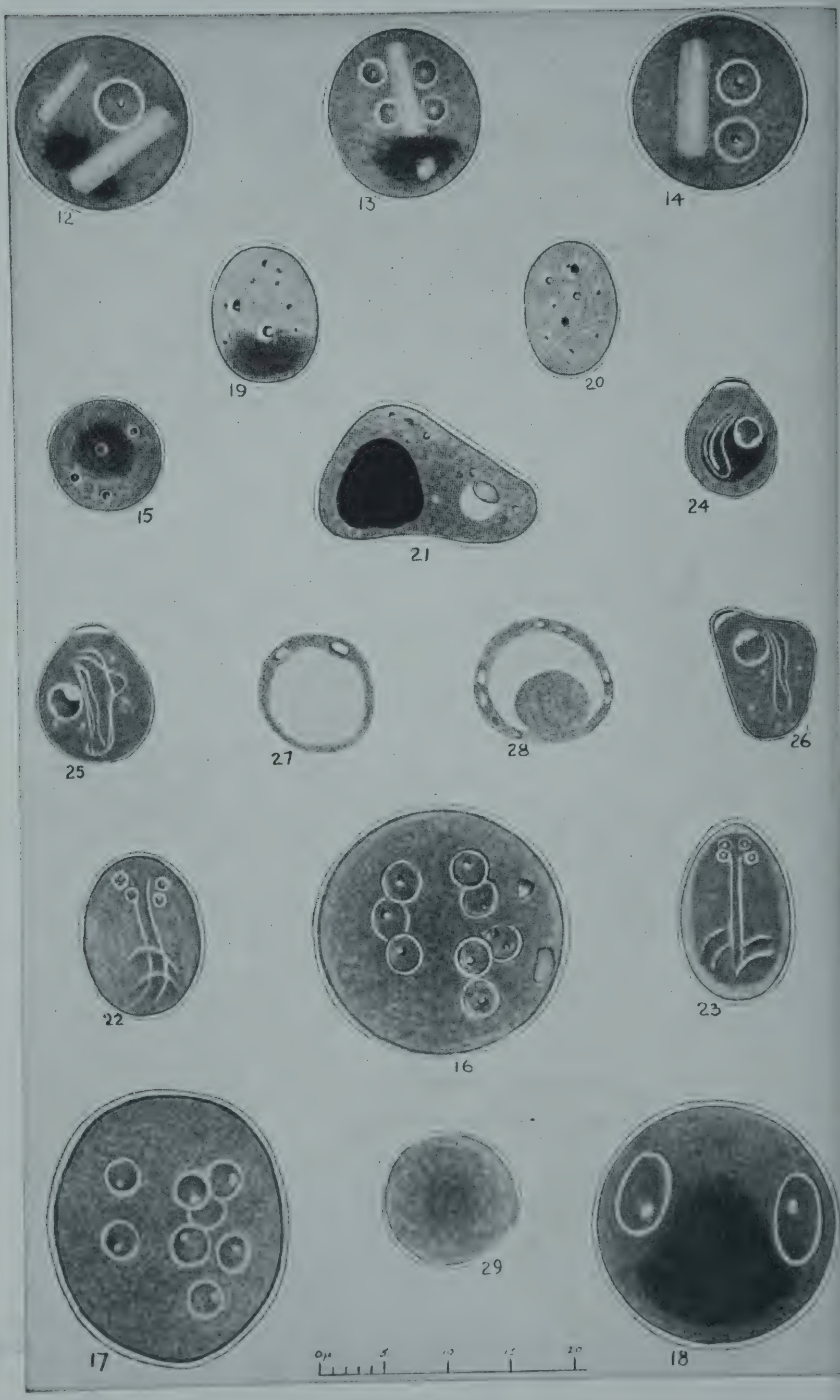
Blastocystis hominis (Fig. 14.4 [figs. 27, 28]; Fig. 14.2 [fig. 31]) is a yeast which is frequently present in human feces and must be differentiated from *E. histolytica* cysts. Its measures from 5 to 15 micra in diameter. In unstained preparations it has a refractile periphery and a large central vacuole. With the iodine stain the peripheral portion stains yellow and usually shows a few colorless granules. The central vacuole is unstained or contains a pale yellow mass.

Other smaller yeasts may be confused with cysts of the smaller amebae or flagellates. They can be identified by their thicker cell walls and coarsely granular contents.

Interpretation of Amebic Infections

If *E. histolytica*, in either motile or cyst form, is found in the stools, it should be taken as presumptive evidence that the wall of the intestine is being invaded, even though no clinical symptoms are present. Amebic liver abscess sometimes develops without a previous history of dysentery or significant intestinal symptoms. Vague abdominal symptoms or general symptoms of a psychoneurotic type may be due to infection with

FIG. 14.4



E. histolytica. In addition, the asymptomatic carrier is the most potent source of transmission of this disease. Therefore, every patient harboring this parasite should receive thorough treatment and should have repeated stool examinations for several months after treatment to be sure of a cure. The diagnosis of infection with *E. histolytica*, however, should be made only on the basis of *accurate* identification of the parasite. Diarrhea, or blood and mucus in the stools, may be due to a variety of causes. Carcinoma of the colon, for instance, has been treated as amebiasis and the chance for operative cure lost by the consequent delay. Dientamoeba is sometimes associated with a diarrhea which disappears after treatment. The other intestinal amebae are non-pathogenic. It should be emphasized that *E. coli* infection is harmless and about twice as prevalent as *E. histolytica*. The laboratory differential diagnosis is difficult, requiring special skill, training, and experience. Untrained and inexperienced workers too frequently identify all amebae as *E. histolytica* or miss these parasites entirely. The results of a cooperative survey by the American Society of Tropical Medicine and the U. S. Public Health Service show that the wise selection and careful performance of laboratory techniques, combined with specialized training and experience of the laboratory worker, contribute markedly to the proficiency of the laboratory.

Serology

Amebic liver abscess too frequently occurs with little or no previous symptoms of intestinal amebiasis and with very few or no parasites present in the stools despite repeated examinations. In such cases the complement fixation test has its greatest value and fortunately is generally positive. However, the incidence of false positive complement fixation tests also is apparently high, so that every positive report must be carefully evaluated by the physician in light of the clinical findings.

FIG. 14.4. Cysts of intestinal protozoa and certain vegetable cysts from fresh stool preparations stained with iodin.

Magnification × 1,860.

Reproduction of Plate 3 in Hygienic Laboratory Bulletin 133, United States Public Health Service, October 1923, "Studies on various intestinal parasites (especially amoebae) of man" by William E. Boeck and Charles Wardell Stiles.

Figs. 12–15. Cysts of *Endamoeba histolytica*.
Figs. 16–18. Cysts of *Endamoeba coli*
Figs. 19, 20. Cysts of *Endolimax nana*
Fig. 21. Cyst of *Iodamoeba bütschlii*
Figs. 22, 23. Cysts of *Giardia lamblia*
Figs. 24–26. Cysts of *Chilomastix mesnili*
Figs. 27, 28. *Blastocystis hominis*, a blastomycete
Fig. 29. A phycomycete

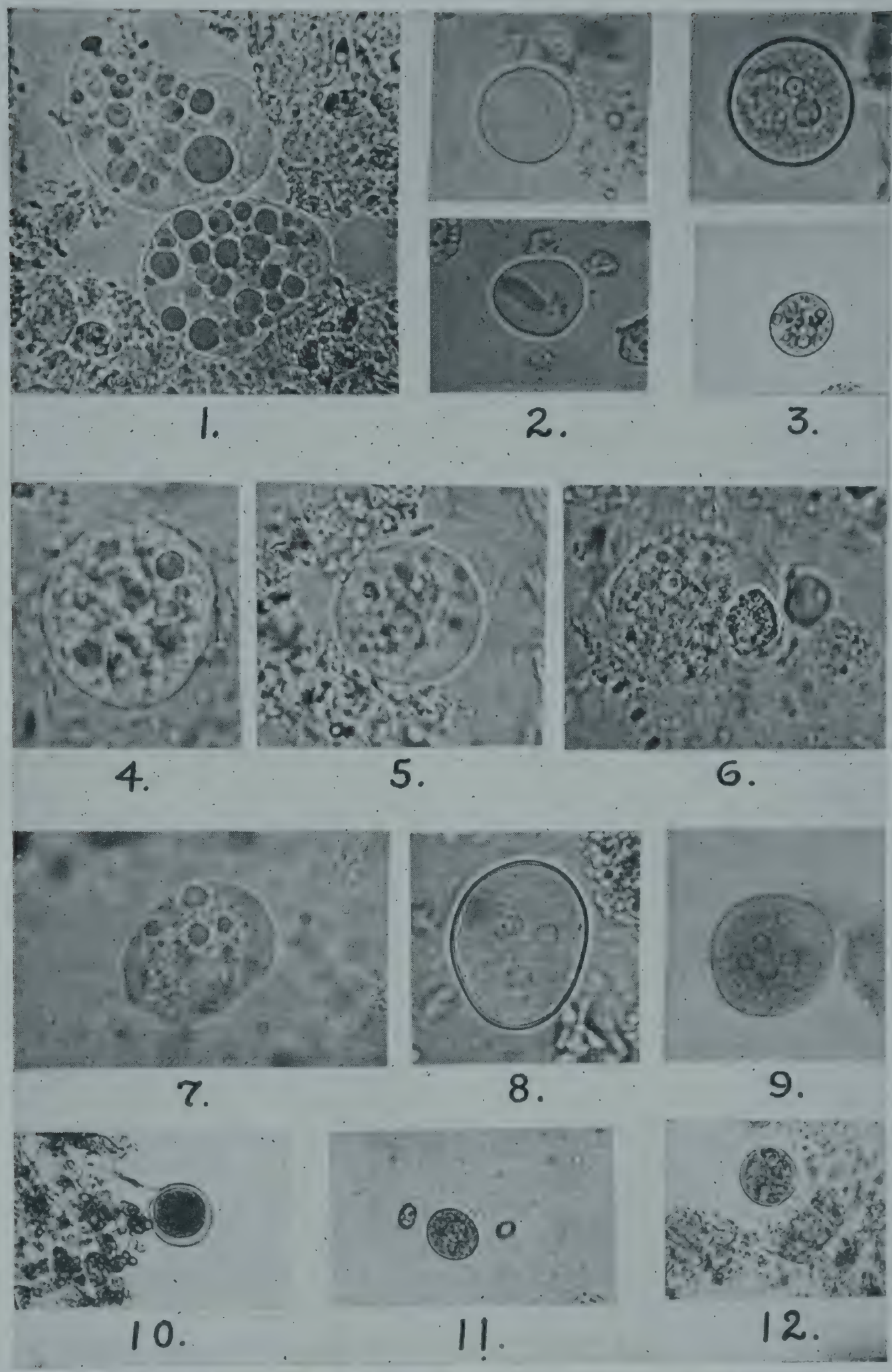


FIG. 14.5 Photomicrographs of trophozoites and cysts of amoebae. (× 750). *1*, *4* and *5*, trophozoites of *Endamoeba histolytica*. *1*, active amoebae containing many red blood cells. *4* and *5*, degenerating forms. *6*, large mononuclear leucocyte simulating amoeba. Note prominence of large oval nucleus and indistinct cell outline. *2*, *3*, *10*, *11*, and *12*, cysts of *E. histolytica*. *2*, unstained cysts. Note the indistinctness of nuclei. Lower figure shows large chromatoidal body. *3*, 4-nucleate cysts of large and small races stained with iodin. Some nuclei are not in focus. Upper figure shows large indistinct glycogen mass. *10*, dead cyst showing shrinking of contents away from cyst wall. *11*, 1-nucleate cyst, small race. *12*, 2-nucleate cyst, small race. *7*, trophozoite of *Endamoeba coli*, small race. Usually *E. coli* is larger than *E. histolytica*. Circular inclusions are vacuoles. *8* and *9*, 8-nucleate cysts of *E. coli*. *8*, unstained. Note that nuclei are visible and four are in focus. *9*, stained with iodin. Six nuclei are in focus.

TABLE 14.4

Differential Features of Amebic Cysts in Iodine Preparations

	Size	Shape	Color iodine stain	Cyst wall	Glycogen vacuoles	Nuclei		
						Number	Membrane	Karyosome position
	μ							
Endamoeba histolytica	6–15	Usually spherical	Yellow	Distinct but thin	If present, reddish brown, usually indistinct	1–4	Distinct with plaques of chromatin, spotty or crescentic	Central, small
Endamoeba coli	15–30	Spherical, oval or irregular	Yellowish brown	Distinct and thicker	If present, dark brown, usually a large central mass	1–8	Thicker, with chromatin more evenly distributed	Eccentric, small or large
Endolimax nana	5–14	Usually ovoidal, may be spherical	Pale green	Thin	Not common, usually darker than in *E. histolytica*	1–4	Thin and indistinct	Eccentric, large
Iodamoeba bütschlii	8–14	Spheroidal or irregular	Greenish yellow	Distinct but thin	Regularly present; a single distinct dark brown mass	1 (rarely 2)	Thin but distinct	Eccentric, large

Unfortunately, the uncomplicated cases of *E. histolytica* infection too frequently show a negative complement fixation reaction for this test to have any value in ruling out infection by this parasite or assisting in the differential diagnosis between *E. histolytica* and *E. coli*. Serology therefore is of greatest assistance in diagnosing extra-intestinal lesions of *E. histolytica* infection.

THE INTESTINAL AND GENITAL FLAGELLATES

As previously pointed out, there are seven parasitic flagellates which are common inhabitants of the intestinal and genital tracts. The incidence of intestinal flagellates roughly parallels the non-pathogenic amebae. *Giardia lamblia*, the most pathogenic of the intestinal flagellates, runs about 7 to 8 per cent, with infection in children three times more prevalent than in adults; *Trichomonas hominis*, 5 to 6 per cent; *Chilomastix mesnili*, 4 to 5 per cent; *Enteromonas hominis*, 3 to 4 per cent; and *Embadomonas intestinalis*, about 1 per cent. The genital flagellate *Trichomonas vaginalis* has a tremendously varying incidence in many surveys, but probably is around 25 per cent in females and something less than 10 per cent in males. *Trichomonas tenax* occurs in about 17 per cent of mouths.

Except for *Giardia lamblia* and *Trichomonas vaginalis*, these parasites are non-pathogenic. Their transmission is directly from man to man, no other animal hosts having been identified. Also, like the amebae, most of them produce cysts which are capable of survival for days outside the body. *T. hominis*, *T. vaginalis*, and *T. tenax* do not produce cysts and all infections are due to transfers of their motile vegetative forms.

These flagellates are rather smaller than most of the amebae, ovoid or pyriform shaped, and have a jerky movement due to the lashing of their flagella. All three Trichomonads possess an undulating membrane in addition to flagella, which gives them a rotary motion. *Giardia lamblia* has the most flagella (8) while *E. intestinalis* has only 2 (1 anterior and 1 posterior). *T. vaginalis* has all 4 of its flagella located anteriorly. For further details, consult Table 14.5.

Diagnostic Techniques

The collection of the specimen, making of the preparation, concentration, and staining techniques are the same as just described for the intestinal amebae. *T. vaginalis* produces no cysts and deteriorates rather rapidly, 20 to 30 minutes, upon drying and exposure to oxygen

TABLE 14.5

Differential Features of Intestinal and Genital Flagellates

	Length	Shape	Flagella	Undulating Membrane	Axostyle	Cytostome
	μ					
Trichomonas hominis.....	11	Pyriform	5	Yes	1	Yes
Trichomonas vaginalis.....	17	Pyriform	4	Yes	1	Faint
Trichomonas tenax.....	10	Pyriform	4	Yes	1	Yes
Chilomastix mesnili	13	Pyriform	4	No	0	Yes
Embadomonas intestinalis..	6	Ovoid	2	No	0	Yes
Enteromonas hominis.....	8	Ovoid	4	No	0	No
Giardia lamblia.....	14	Pyriform	4–8 (variable)	No	0	No

and room temperature, so moist vaginal smears should be examined soon after preparation. *T. hominis*, however, will survive 24 hours in feces.

Symptoms

The symptoms of *Giardia lamblia* infection in children are confined to the diarrhea syndrome. In adults, the diarrhea syndrome may be less pronounced and symptoms similating biliary disorders more prominent.

Trichomonas vaginalis infection produces in many females a profuse irritating leukorrhea with burning, itching, and a yellowish-creamy sero-purulent discharge. Infection in the male generally produces so few symptoms that the infected individual is unaware of his infection. On occasion, perhaps with secondary bacterial invaders, a low grade chronic urethritis or prostatitis may be produced with a thin, scanty, watery to purulent discharge.

Identification of Intestinal Flagellates

The identification of intestinal flagellates depends upon recognition of the characteristic motile vegetative forms or identification of their cysts, except in the case of *T. hominis* which produces no cysts. Cultures are of no assistance, for *Giardia lamblia* has never been cultivated and it is the medially important member. No serological tests are available; therefore, microscopical demonstration of the motile vegetative forms or cysts is the only diagnostic means available. Using fresh material and following the techniques described for the intestinal amebae it is not too difficult to identify the motile vegetative forms (see Table 14.5). Using direct smears and concentration techniques the cysts also can be dem-

TABLE 14.6

Distinguishing Characteristics of Intestinal Flagellate Cysts in Iodine Preparations

	Length	Flagella	Nuclei	Spiral Groove	Color with Iodine
	μ				
Giardia lamblia	11	+	Usually 2–4	No	Greenish-yellow
Chilomastix mesnili	8	+	1	Yes	Yellow; lighter anterior end
Enteromonas hominis	7	0	1–4, usually 2	No	Yellow
Embadomonas intestinalis	5	0	1	No	Yellow

onstrated. Their identification requires considerable practice and experience. See Table 14.6 for distinguishing characteristics. In iodine preparations, the cysts of *Giardia lamblia* are oval structures of about 12 micra, with a well-developed cyst wall from which the contents often are separated by a clear space. Usually the 4 dark-rimmed nuclei, 2 at each end, can be distinguished. There are distinct longitudinal lines representing the flagella, while the cyst has a greenish-yellow color.

Chilomastix mesnili. These cysts are smaller, broadly pear-shaped, about 8 micra in length, and containing only 1 fairly large nucleus. The flagella are less distinct and the color is yellow, lighter at the anterior end.

E. hominis. These cysts are still slightly smaller, measuring 6 to 8 micra in length. There are usually 2 nuclei, one at each end, and the cytoplasm may show small round refractile bodies. The color is yellow.

E. intestinalis. These cysts are small, 5 or 6 micra, pear-shaped bodies containing a single nucleus with a central karyosome.

Identification of Genital Flagellates

Trichomonas vaginalis produces no cysts, and although it may be cultivated on a number of media it is much simpler to identify the organism in fresh smears. No serological tests exist. When smears cannot be examined at once, they may be stained. *T. vaginalis* shows up well with ordinary Wright or Giemsa stains.

The fresh wet smear is the simplest and best diagnostic preparation. The organisms are usually so abundant in untreated cases that the identification is easily made. *T. vaginalis* is the largest of the trichomonads, and in unstained moist preparations they appear as colorless pyriform or, at times, oval organisms with four anterior flagella and an undulating membrane extending back one-half to two-thirds of the body.

The organisms are actively motile with a somewhat rotary motion due to the combined activity of the flagella and undulating membrane. There are vacuoles present and a single oval nucleus at the anterior end.

Interpretation of Flagellate Infections

The pathogenicity of intestinal flagellates is much debated. There is evidence that in some individuals they are the cause of a watery or mucous diarrhea, and that Giardia may give rise to indefinite upper abdominal symptoms. These symptoms are often associated with enormous numbers of motile flagellates in the stool, and the number of flagellates greatly diminishes with the disappearance of symptoms. On the other hand, *Trichomonas hominis* may be present in the intestine in large numbers without producing diarrhea or other symptoms. Also, many Chilomastix or Giardia cysts may be found in stools of apparently normal individuals. From a clinical standpoint, it is justifiable, if symptoms are present in Trichomonas and Chilomastix infections, to administer one of the newer anti-amebic drugs since these parasites can sometimes be eradicated by such treatment. Giardia can usually be eliminated by quinacrine (atabrine). The diarrhea, however, may be due to some other cause which should be searched for. The diet should be carefully regulated and every effort made to ascertain the cause of the intestinal complaints.

Trichomonas vaginalis infections should be energetically treated to improve the vaginal hygiene, and specific medications used to eradicate the *T. vaginalis* organisms and restore the normal vaginal pH and flora. Infections in the male are perhaps best treated by atabrine.

CILIATES

One ciliated protozoan, *Balantidium coli*, inhabits the intestinal tract and may produce serious disease. This parasite is a normal inhabitant of the intestinal tract of hogs and rarely of man. The disease has been called Balantidiasis or Balantidial dysentery. *Balantidium coli* is the largest protozoan inhabiting the intestinal tract. The oval trophozoites measure 50 to 70 micra in length and 30 to 40 micra in width. The surface is covered with longitudinal rows of cilia. At the anterior end near the mid line there is a narrow opening, the cytostome. The macronucleus, large, elongated and kidney-shaped, is centrally located and in its concavity there is a small micronucleus. Constant rhythmic motion of the cilia produces an active and slowly rotary motility that appears particularly adept at penetrating the intestinal mucosa. Under adverse

conditions in the lower colon and rectum, trophozoites secrete a thick wall, forming oval cysts 45 to 65 micra in diameter; the trophozoites soon lose their cilia. These cysts have a greenish-yellow appearance in iodine preparations. The macronucleus is retained, but food vacuoles disappear.

Symptoms

Severe infections produce acute diarrhea or dysentery that may be fatal. However, the usual infections are more superficial, yield milder symptoms or, in the carrier stage, few or no symptoms. The asymptomatic carrier is more common than the acute case. The anti-amebic drugs have varying degrees of efficacy; however, Terramycin and some of the newer antibiotics appear quite effective.

Identification of Balantidium coli

Fecal smears are made as for other intestinal protozoa and the vegetative form is easily identified because of its relatively large size and striking physical characteristics. The cysts can be found by the zinc sulphate flotation technique. Stained smears can also be examined for the cysts which, however, are less strikingly characteristic. No serological tests have been divised, and although the organisms may be cultured, direct microscopic examination of smears is the simplest and most reliable.

Protozoa Inhabiting the Skin, Blood, or Internal Organs—The Hemoflagellates

Although *E. histolytica* may cause chronic skin ulcerations, particularly in constantly moist warm climates, the chief protozoa inhabiting the skin, blood, or internal organs belong to either the Mastigophora, the hemoflagellates, or Sporozoa, which are non-motile spore-forming organisms.

The hemoflagellates live in the skin or blood or internal organs. There are six medically important hemoflagellates belonging to two genera—three Trypanosoma and three Leishmania (see Table 14.7). The typical trypanosome has a variable elongated, tapering, curved body with a single vesicular nucleus. There is a single flagellum extending out anteriorly as a free portion. The attached portion along the body is regarded as an undulating membrane.

TRYPANOSOMES

The life cycles of these hemoflagellates are more complicated than any we have so far encountered. Here for the first time we find the

TABLE 14.7

Protozoa Inhabiting the Skin, Blood, and Internal Organs

Name	Disease	Organ Location
Mastigophora (flagellates)		
Trypanosoma gambiense	West African sleeping sickness	Blood, lymphatic and central nervous system
Trypanosoma rhodesiense	East African sleeping sickness, South and Central American sleeping sickness	Blood, lymphatic and central nervous system
Trypanosoma cruzi	Chagas' disease	Skin and internal organs
Leishmania donovani	Kala-Azar	Spleen, liver, and bone marrow
Leishmania brasiliensis	Espundia, mucocutaneous leishmaniasis	Mucocutaneous
Leishmania tropica	Oriental sore	Mucocutaneous
Sporozoa (non-motile spore forming)		
Toxoplasma gondii	Chorioretinitis, encephalomyelitis	Eye and central nervous system
Plasmodium vivax	Tertian malaria	Erythrocytes and liver
Plasmodium malariae	Quartian,malaria	Erythrocytes and liver
Plasmodium falciparum	Estivo-autumnal malaria	Erythrocytes and liver
Plasmodium ovale	African tertian malaria	Erythrocytes and liver

necessity for two separate hosts. One, an invertebrate, serves as the intermediate host and man as the final host. After inoculation into man there is a rapid multiplication by longitudinal fission. *Trypanosoma gambiense* and *T. rhodesiense* invade the blood, lymphatics, and finally the central nervous system. *T. cruzi* tends to localize more in the skin, voluntary muscle, myocardium, and brain, with fewer organisms in the blood and lymphatics. In man, *T. gambiense* and *T. rhodesiense* produce African sleeping sickness, while *T. cruzi*, the American trypanosome, produces Chagas' disease. These latter organisms, while multiplying within a muscle cell, assume a small oval Leishmania-like shape, and large numbers develop in cyst-like spaces. Finally, they elongate and the cyst ruptures, freeing the trypanosomes for further blood stream and tissue invasion.

T. rhodesiense and *T. gambiense* infections are transmitted by the bite of the tsetse fly, which serves as the invertebrate host. Following the

TABLE 14.8

Differential Characteristics of Three Types of Trypanosomes

	T. gambiense and *T. rhodesiense*	*T. cruzi*
Length (μ)	15–30	20
Shape	Polymorphic	Sharply curved
Position of nucleus	Central, rarely posterior to center (rhodesiense)	Central
Blepharoplast	Small dot	Large oval
Flagellum	Long, short, or absent	$\frac{1}{3}$ length of body

ingestion of infected blood by the fly, the parasites multiply in the gut. After two weeks, long slender forms are produced that migrate to the salivary glands and ducts. Here a further development takes place with the production of the infective "metacyclic" parasites, which are then transmitted by the bite of the fly to man and other vertebrate hosts, starting the disease cycle over again.

T. cruzi infection in South and Central America is transmitted by an arthropod vector, the reduviid bug, a Triatoma, although not by its bite but rather by its infected feces.

Identification of Trypanosomes

Trypanosomes inhabiting the blood and central nervous system may be demonstrated in moist peripheral blood smears but are best seen in a darkfield preparation. However, they are never very numerous in the blood, so best results are obtained by concentrating the parasites present in 10 ml. of citrated blood by centrifugation. Stained smears of the sediment will show the parasites. When direct demonstration of the parasites fails, guinea pigs and/or rats may be inoculated with blood or spinal fluid sediment. In fully developed cases of sleeping sickness, stained smears of sediment of centrifuged spinal fluid will also show these parasites. A final technique that has been used successfully when the parasites are too rare for demonstration in the peripheral blood is to allow laboratory-bred triatomids to bite the patient. If the infection is present the parasites multiply rapidly in the intestines of the triatomids and can be easily demonstrated by examination of the intestinal contents.

Trypanosomes inhabiting the skin and internal organs frequently can be demonstrated in stained smear preparations made by aspiration biopsy of enlarged lymph nodes, spleen, or sternal bone marrow. Macer-

ated lymph gland material can be inoculated into guinea pigs and the infection demonstrated in these animals by repeated examination of their peripheral blood.

Interpretation of Trypanosome Infections

Human trypanosome infections may remain asymptomatic for weeks or months. Symptoms are gradual in onset and the characteristic clinical picture is late in development. American trypanosomiasis is practically limited to natives of the endemic region, since infection usually is acquired in huts of poor construction harboring the vector, a cone-nosed Triatoma. African trypanosomiasis should be kept in mind in persons who have visited the endemic region. The tsetse fly is no respector of persons. In suspected cases a negative blood examination should be supplemented by lymph-node puncture and spinal fluid examination.

LEISHMANIA

The Leishmania belong to the trypanosome family. They exist in man as small oval non-flagellate forms (Leishman-Donovan bodies), but in insects or in culture at 20°C. they become flagellates without an undulating membrane and with the kinetoplast at the anterior end (leptomonads). In man there are two main clinical types of leishmaniasis, the cutaneous or local (tropical sore, espundia), and the visceral or systemic (kala-azar). Three species, morphologically identical, are recognized: *Leishmania tropica* and *Leishmania brasiliensis*, causing cutaneous lesions, and *Leishmania donovani* causing a systemic kala-azar. Leishmania are primarily parasites of the endothelial cells and the large mononuclear phagocytes of the body (macrophages, clasmatocytes, Kupffer cells). They multiply in these cells until the cells burst. If such cells are associated with the blood stream, Leishmania may be found free in the blood plasma or in circulating cells. This occurs only in the visceral type, kala-azar with rupture of parasitized capillary endothelial cells.

The Leishman-Donovan bodies (Fig. 14.6 and Fig. 14.7 [fig. 9]) are oval, 2 to 5 micra in length. Stained by Wright's method they show a delicate cell membrane, faintly blue-staining cytoplasm, a purple eccentric nucleus and a minute, densely purple-staining, rod-shaped kinetoplast which is usually directed radially away from the nucleus. The cytoplasm and cell membrane may not be visible, but the nucleus and kinetoplast together are diagnostic.

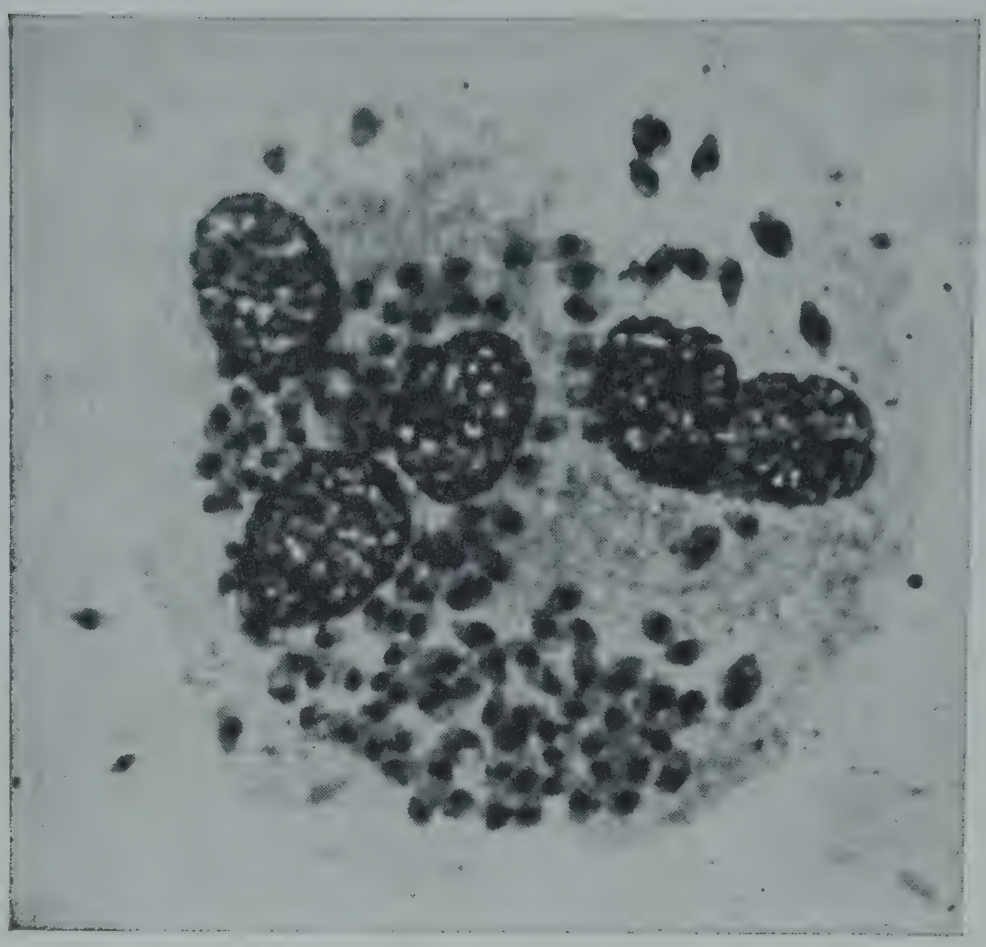


FIG. 14.6. *Leishmania tropica* in smear, × 1500. (Photo by Edgar Nebel, Dept. of Pathology, N. Y. U.)

Diagnostic Methods for Leishmania

In visceral leishmaniasis (kala-azar) the procedures for finding the parasites are, in the order of their efficiency: spleen puncture, bone marrow puncture, lymph node puncture, peripheral blood examination. There is little danger from spleen puncture if it is correctly performed and if the patient's clotting and bleeding time are not prolonged. These factors should be determined, and if prolonged they should be brought within normal limits by transfusion before spleen puncture is performed.

Technique of Spleen Puncture. The spleen should be easily palpable below the costal margin. An 18-gauge needle, $1\frac{1}{4}$ inches long, perfectly fitted to a 5-ml. or 10-ml. syringe, is the most suitable intrument. Needle and syringe should be dry sterilized. Sterile gloves are worn. The point for puncture should be at least one-half inch both from the spleen edge and from the costal margin. After sterilizing the skin, the point of puncture is anesthetized with novocaine. The patient is instructed to remain quiet while the puncture is performed. An assistant places one hand below the lower edge of the spleen. The operator inserts the needle gently but firmly in two movements, first vertically through the anesthetized skin, and second upward and outward into the spleen. He then exerts strong suction on the plunger of the syringe quickly two or three times, then releases the plunger and quickly withdraws the needle. It is not desired to draw blood into the syringe. An abdominal binder should be applied and the patient instructed to lie in bed quietly for a few hours. He should be observed for possible signs of internal hemorrhage, though the danger of this is minimal.

The spleen pulp drawn into the needle can be treated in several ways. (1) It can be expelled on to a glass slide, smeared thinly and stained with a blood stain. (2) It can be diluted with a few drops of sterile saline and distributed at the base of several slants of N.N.N. medium for incubation *at room temperature* to produce development of the leptomonad forms of Leishmania. (3) It can be diluted with 1 ml. of sterile saline and injected intraperitoneally into two hamsters. These animals may develop an infection in which L-D bodies can be found in spleen smears after 4 to 6 weeks. A combination of the above procedures is most desirable.

Technique of Bone Marrow Puncture. See Chapter 2. Specimens are handled as in spleen puncture.

Technique of Lymph Node Puncture. An enlarged node can be used. A 20-gauge needle on a 2-ml. syringe is suitable. After the skin has been sterilized and anesthetized the lymph node is grasped between the gloved fingers, the needle is thrust into it and suction is applied while the needle point is moved about gently in the node. The aspirated material is handled as in spleen puncture. A lymph node removed for microscopic sectioning may show Leishman-Donovan bodies. Smears from the cut surface of such a lymph node should also be made and stained with a blood stain for L-D bodies.

Examination of Blood for Leishmania. Leishman-Donovan bodies sometimes may be found in ordinary thin blood films in advanced cases, usually in the cytoplasm of large mononuclear leukocytes, less commonly outside of cells. They may be found more readily in dehemoglobinized thick-film preparations, but if not within cells, they must be carefully differentiated from blood platelets. They may also be found by centrifuging 5 to 10 ml. of citrated blood at high speed. The parasitized cells are heavier than the other blood cells, and are therefore more numerous at the very bottom of the tube. A small drop of blood cells removed from this location is spread thinly on a slide and stained with Wright's stain.

Additional Aids in Diagnosis of Visceral Leishmaniasis.

(1) Leukopenia. The leukocyte count becomes progressively decreased and may reach 1500 w.b.c. per ml. The decrease is mainly in neutrophils.
(2) Anemia appears later in the disease, is secondary in type and moderate in degree.
(3) Thrombocytopenia develops in advanced cases.
(4) Blood serum globulin becomes gradually increased, often to 10 gm. per cent, with inversion of albumin:globulin ratio.
(5) Aldehyde test parallels increase of blood globulin. To 1 ml. of patient's serum in a test tube add 1 to 2 drops of strong formalin. Shake and allow to stand. Formation of an opaque white jell is a positive reaction.

Interpretation of Leishmania Infections

Leishmaniasis is not endemic in the United States and is not likely to become established because the sand-fly, Phlebotomus, some species of which act as the vector, is present only in a few restricted areas in this country and, moreover, imported cases are rare. However, occasional cases are encountered in our port cities, and several scores of cases were contracted by our troops in the Mediterranean area and in

India. Since the incubation period may be as long as fifteen months, some cases may develop in veterans after discharge from military service.

The conditions most often confused with visceral leishmaniasis are other splenomegalies such as chronic malaria and aleukemic leukemia. In the United States several cases of histoplasmosis have been misdiagnosed as kala-azar.

Cutaneous leishmaniasis can be mistaken for fungous or other chronic granulomatous lesions of the skin. The two principal endemic areas are the Middle East and Central and South America. Although the classical American type is a destructive lesion of the skin and mucous membranes, many cases, especially in Central America, resemble the less ulcerative lesions of the Middle East.

Sporozoa (The Nonmotile Spore-Forming Protozoa)

The class Sporozoa contains 5 organisms that produce infections in man, *Toxoplasma gondii* and the 4 malarial parasites (see Table 14.7). Although *T. gondii* has not been proven definitely to belong to this class, it is being arbitrarily placed here pending further work.

TOXOPLASMA GONDII

This is a crescent- to oval-shaped parasite, 4 to 8 micra in length, which appears much smaller in fixed tissue sections. It has a thin cell wall and the nucleus is located toward the rounded end. The parasites are often found in clusters within cells and appear to have a predilection for reticulo-endothelial cells. Multiplication takes place by longitudinal fission. The life cycle as yet is not known; however, it has been found in a variety of the smaller vertebrate animals producing in many a severely destructive disease. In human infections the organism appears to have a predilection for the eye and central nervous system, although in the acute disease the organisms invade almost every tissue in the body. In subacute cases, the greastest destructive lesions take place in the brain and eye, and children are most severely affected. In adults chorioretinitis due to this infection has been demonstrated and the organism isolated from such a case by Jacobs *et al.* Apparently many asymptomatic cases exist, and females can transmit the infection to their babies. Such congenitally acquired infections are now being increasingly recognized by early eye and central nervous system involvement.

Identification of Toxoplasma gondii

The organisms can be demonstrated in smears, biopsy, and autopsy tissues, and by animal inoculation. Body fluids should be centrifuged and smears made of the sediment using Giemsa's or Wright's stain. Tissue sections stained with hematoxylin and eosin show the organisms within parenchymal cells. Blood, spinal fluid, body fluids, and ground-up tissue can be injected into mice and guinea pigs. Complement fixation and "dye tests" have been developed as well as skin test antigens that give promise of becoming good diagnostic tools—particularly the dye test and skin test.

THE PLASMODIA-MALARIA PARASITES

The remaining four Sporozoa producing infections in man all belong to the genus Plasmodium. They are the human malarial parasites. Their life cycles are quite similar: each requires an invertebrate—the Anopheles mosquito—as definite host, where the sexual cycle occurs; each requires man as the intermediate host, where the asexual cycle takes place. The four malarial parasites and their diseases are:

TABLE 14.9

The Malaria Parasites

Name	Type of Malaria
Plasmodium vivax	Benign tertian or vivax
Plasmodium malariae	Quartan
Plasmodium falciparum	Estivo-autumnal or malignant tertian
Plasmodium ovale	Benign tertian (African)

Life Cycle

These parasites require two hosts for their complete life cycle—man and a female Anopheles mosquito (only the females bite). The development of the asexual cycle in man is termed *schizogony* and the sexual cycle in the mosquito, *sporogony*.

Schizogony. An infected female Anopheles mosquito, when biting man, transmits from its salivary glands long slender motile organisms, the *sporozoites.* These are 10 to 12 micra long and 1 or 2 micra wide. They enter the blood stream probably seeking out the parenchymal cells in the liver, where apparently they multiply and develop into small

oval bodies called micromerozoites which, upon completion of this exo-erythrocytic stage, return to the blood stream and enter red blood cells. When a *micromerozoite*, a young *trophozoite*, enters an erythrocyte the cycle of *asexual schizogony* occurs. In the erythrocyte, the malarial trophozoite is a small blue-staining mass of cytoplasm with a red-staining so-called "signet-ring" dot or nucleus. A large central vacuole soon develops, and as it grows the parasite becomes irregular, the nucleus divides, and pigment begins to form. This developing trophozoite is termed a *schizont*, and when fully developed it segments and splits up into a number of individual *merozoites*. The red blood cell upon which the schizont has been feeding gradually enlarges, and following the segmentation the cell ruptures and thus is destroyed. Rupture of the cell liberates these new individual parasites, the merozoites. The free merozoites immediately attack new erythrocytes, entering the cell and repeating the cycle. The time of development of a single trophozoite into a mature schizont, its segmentation with the production of individual merozoites, and rupture of the red blood cell varies with the species of malaria parasite. As schizogony becomes well established for reasons as yet unknown a few of these merozoites, upon entering an erythrocyte, develop into sexual forms, the male and female *gametocytes*. The male gametocyte, being smaller, is termed a microgametocyte and the larger female form, the macrogametocyte. However, these sexual forms, *gametocytes*, are incapable of reproduction while within man. They can reproduce only in the stomach of a female Anopheles mosquito.

Sporogony. When a female Anopheles mosquito, by biting an infected individual, ingests blood containing micro- and macrogametocytes, the sexual cycle, *sporogony*, ensues. The *microgametocyte* divides into a number of fine slender filaments, a process called *exflagellation*. These fine filamentous *microgametes* seek a female gamete which has prepared for fertilization by extrusion of a portion of its nucleus. Only one microgamete enters a female *macrogamete*, the resulting fusion of their nuclei being termed *syngamy*, and a new cell, the *zygote*, results. The *zygote* immediately elongates and becomes motile and this form is termed an *oökinete*, which migrates into the stomach wall and develops into an oval cyst, the *oöcyst*. Within this *oöcyst* the nuclear material divides multiple times, ultimately producing by *sporogony* thousands of *sporozoites* which are released into the body cavity of the mosquito by rupture of the *oöcyst*. The motile sporozoites migrate to the salivary glands and ducts of the mosquito and then the mosquito becomes infective for man.

Sporogony takes place in about 10 days to 2 weeks, depending upon the species and temperature.

The epidemiology, prevention, clinical diagnosis, and treatment will not be considered here—only the laboratory diagnosis.

Identification of the Malaria Parasites

From the foregoing description it is readily apparent that demonstration of the malaria parasites in the blood of an infected individual is the simplest method of laboratory identification of these parasites. Malaria parasites may be few or many in the peripheral blood. A very thin film in which the red blood cells do not overlie each other is best for accurate diagnosis, but if the parasites are few they may be found only in thicker areas of the film. Larger forms may be located by the high dry objective with oil on the slide, but only the oil-immersion objective can be depended upon for identification. If the parasites are very few in number, a dehemoglobinized thick film may be necessary.

Technique for Thick Blood Film Diagnosis of Malaria. Only new slides should be used. They should be washed with soap and water, rinsed in distilled water and alcohol, and dried with a lint-free cloth. The patient's finger should be cleaned carefully, pricked deeply, and held upright. The slide should be touched to the drop of blood but not to the finger and the blood spread by a rotary motion of the slide to an area about 2 cm. in diameter. To insure the blood sticking to the slide some workers emphasize the value of stirring with a rotary motion, using the pricking needle for a few seconds. By tilting the slide more blood can be added to the shallow portion of the drop. The final drop should be of medium thickness, opaque but only slightly raised. It should be allowed to dry in an upright horizontal position without being disturbed and protected from insects and dust. When thoroughly dry, preferably after several hours, it should be stained with diluted Giemsa stain of certified quality.

The staining of numerous thick blood films together by mass procedures may be a dangerous practice, for Brooke and Donaldson have demonstrated that minute bits of the thick blood films frequently flake off from one slide and are deposited on another slide. If, as has happened, flakes from a positive film containing parasites are deposited on a negative film an erroneous diagnosis may result. This danger can be eliminated by the addition of 0.5 per cent of a surface-active agent such as Triton X-30, which does not interfere with the staining.

This method saves much time and increases the chance of a positive diagnosis, but is reliable only if the examiner is familiar with the appearance of the malaria parasites in such a preparation. Since the red cells are destroyed the identification is based entirely upon the appearance of the parasites themselves. Nevertheless this method is essential in sur-

veys for malaria and should be used in hospitals and private practice, particularly in the chronic cases.

Time of Examination

Any time in the cycle of the parasite is suitable for a blood examination for malaria, and it should not be delayed. At the beginning of the chill many parasites have not yet entered new red blood cells. The small ring forms are most numerous from 4 to 8 hours after the chill. Fewer but larger parasites easier to detect are present later in the cycle. In P. falciparum infections (estivo-autumnal malaria) the parasitized red blood cells appear to become sticky after the ring stage is passed and adhere to the walls of small blood vessels in the internal organs, so ordinarily they are not found in peripheral blood smears. For this reason failure to find parasites on one day should never exclude a diagnosis of malaria, and the examination should be repeated the *following* day. Sometimes three or four examinations on consecutive days are necessary before a diagnosis can be made. In rare cases of chronic malaria the parasites may not be demonstrated even by several thick-film examinations, although the case may respond to treatment. The existence of such infections has been proven by inoculation of blood into another individual.

Description of Malaria Plasmodia

The four species of human malaria parasites are *Plasmodium vivax* (benign tertian), *Plasmodium malariae* (quartan), *Plasmodium falciparum* (estivo-autumnal, subtertian, malignant tertian), and *Plasmodium ovale*.

All species of plasmodia have the same characteristics in man. They inhabit the red blood cells and contain pigment (hemozoin) produced from hemoglobin. The appearance of the *trophozoite*, the signet-ring forms, the developing *schizont*, and the number of *merozoites* is characteristic for each species, making possible the exact identification of the *Plasmodium* present. The characteristic malaria chill and fever are caused by the simultaneous rupture of large numbers of red blood cells with the accompanying liberation of vast numbers of merozoites and pigment.

The young trophozoites in red blood cells consist of a small blue-staining mass of cytoplasm and a red dot of chromatin. A vacuole or unstained central area quickly develops, about which is a rim of blue cytoplasm with the chromatin dot at one side. This is called the *signet-*

ring stage. As this grows larger the cytoplasm becomes more prominent and pigment granules begin to appear in it. When the parasite is about half grown the chromatin mass, which has become larger, divides into two masses, and later this division is repeated until the characteristic number for the species is reached. The dividing parasite is called a *schizont*. The pigment becomes gathered together into a central mass and the cytoplasm divides about the chromatin masses forming a sort of "rosette." When this segmentation is complete the red cell ruptures liberating the individual *merozoites*, which penetrate new red cells and repeat the *schizogony*.

The *gametocytes* usually begin to appear in the peripheral blood about one week after the onset of clinical symptoms. They are difficult to recognize before they have become mature. When mature those of *P. vivax* and *P. malariae* are round or oval, are about as large as a mature schizont, have blue-staining cytoplasm, a single chromatin mass, sometimes a small vacuole, and the pigment granules are scattered throughout the cytoplasm. The gametocytes of *P. falciparum* are characteristically banana-shaped and are called "crescents." In all three species the female gametocyte is slightly larger than the male and its cytoplasm stains a deeper blue, but the differentiation of the sexes is difficult without considerable experience.

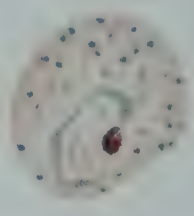
Differentiation of Malaria Plasmodia (Plates 14.I, 14.II, 14.III)

The asexual cycle of *P. vivax* is completed in approximately 48 hours, that of *P. malariae* in approximately 72 hours, and that of *P. falciparum* in 44 to 48 hours. In the signet-ring stage the species are difficult to differentiate, but there are certain suggestive differences. In infections with *P. vivax* and *P. malariae* one often finds older forms (merozoites) at the same time, which may definitely identify the species, whereas in *P. falciparum* infections the older forms, as previously stated, are held, in the small blood vessels of internal organs. The crescent gametocytes however, continue to circulate in peripheral blood and, if present, are easily identified. The ring forms of *P. falciparum* more frequently have two chromatin dots than those of the other two species, and these are frequently located at opposite sides of the ring. The rings of *P. falciparum* are also more frequently located at the edge of the red blood cell, and there are more often several rings in the same red blood cell. The ring forms of *P. vivax* and *P. malariae* are practically indistinguishable from each other.

The growing trophozoites and schizonts of *P. vivax* are larger, more


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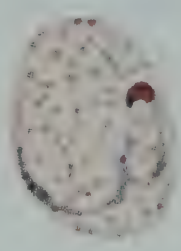

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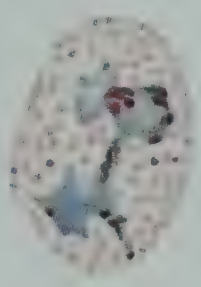

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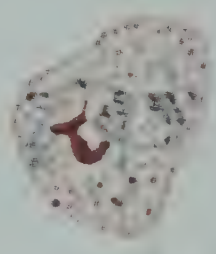

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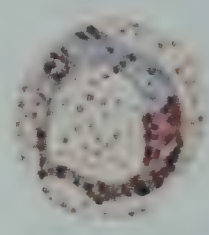

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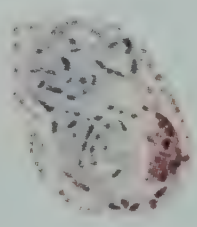

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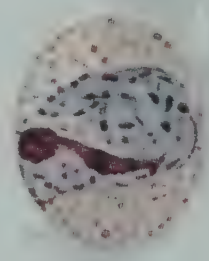

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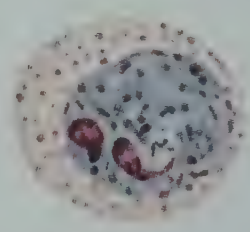

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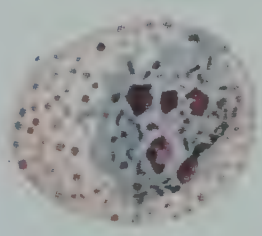

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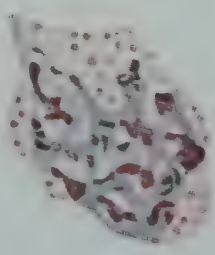

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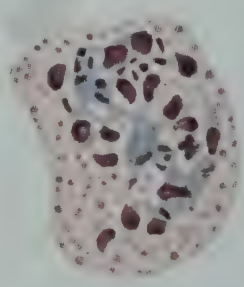

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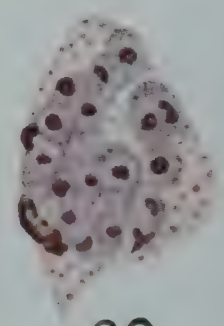

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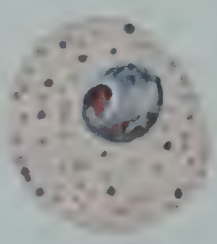

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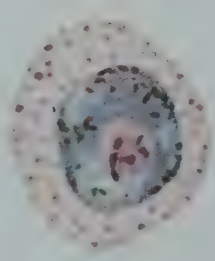

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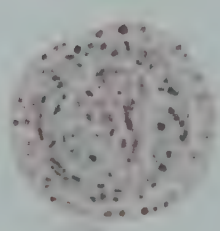

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INEZ DEMONET

irregular in shape and usually a paler blue in color than those of *P. malariae*. Those of *P. malariae* often extend across the red blood cell in a "band form." The pigment of *P. vivax* is in finer particles and of a golden-brown color while that of *P. malariae* is more compact and darker brown. P. vivax develops from 16 to 24 merozoites, *P. malariae* usually 8. The red blood cell containing *P. vivax* becomes definitely enlarged and pale after the signet-ring stage is passed. This is one of the most reliable diagnostic points. If the stain is not too acid these enlarged red blood cells containing *P. vivax* show a sprinkling of fine red or bluish-red dots called *Schüffner's dots*. The red cells containing *P. malariae*, on the other hand, contain no such dots, are often slightly smaller than normal, and may have a bronze color.

Occasionally in very severe or moribund cases of falciparum malaria infections, the larger trophozoites and schizonts may be found in peripheral blood smears. They are about the same size as those of *P. malariae*, are usually circular in shape, and the pigment is in a large compact mass at the center of the parasite. Fully developed schizonts contain 16 to 32 merozoites. The red blood cells are usually pale but are not enlarged. They often contain a few reddish-blue masses called *Maurer's dots*, which are irregular in size and shape and larger than Schüffner's dots.

The crescent gametocytes of *P. falciparum* are often free in the blood stream. If the red cell has not ruptured frequently it is very pale and

PLATE 14.I.—*P. vivax*

1. Normal sized red cell with marginal ring form trophozoite.
2. Young signet ring form trophozoite in a macrocyte.
3. Slightly older ring form trophozoite in red cell showing basophilic stippling.
4. Polychromatophilic red cell containing young tertian parasite with pseudopodia.
5. Ring form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schüffner's stippling.*
6. 7. Very tenuous medium trophozoite forms.
8. Three amoeboid trophozoites with fused cytoplasm.
9, 11, 12, 13. Older amoeboid trophozoites in process of development.
10. Two amoeboid trophozoites in one cell.
14. Mature trophozoite.
15. Mature trophozoite with chromatin apparently in process of division.
16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").
20. Mature schizont.
21, 22. Developing gametocytes.
23. Mature microgametocyte.
24. Mature macrogametocyte.

* Schüffner's stippling does not appear in all cells containing the growing and older forms of *P. vivax* as would be indicated by these pictures, but can be found with any stage from the fairly young ring form onward. (Courtesy U. S. Public Health Service.)


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can be seen only as a delicate line stretching across the concave side of the parasite. The male gametocyte is usually more pointed than the female, is paler blue in color, and its pigment is more scattered.

Table 14.10 summarizes the differential points of the three common species of plasmodia.

In the thick blood film (Plates 14.IV, 14.V, 14.VI), differentiation of the three species is more difficult because the red blood cells have been hemolyzed. Differentiation is easiest in the thin edge of the film where drying has been quickest and where the red cells sometimes are preserved. If only rings are present differentiation is impossible unless the rings are numerous and many of them have two chromatin dots, in which case they are probably *P. falciparum*. If there are large round forms with delicate pigment, they are *P. vivax*. In these forms the Schüffner's dots of the red blood cells are often still visible. *P. malariae* is small and compact with coarse dark pigment. The crescents of *P. falciparum* may round up before the thick film has dried, and therefore may not be characteristic in appearance, but typical ones usually can be found at the thin edge of the film.

Artifacts Mistaken for Malaria Parasites in Thick Films

Bulletin No. 180 of the National Institute of Health gives the following precautions in preparing and examining a thick blood film for malaria.

"The inexperienced microscopist may be confused in the examination of thick films by bacteria or dirt from the skin; by dust particles on the slide; by vegetable spores, yeast cells or fungi from the air; and by bacteria, molds, protozoa, or other contaminants from the distilled water used in staining. Probably the chief source of dirt on the thick film is from the improperly cleaned finger. If the glass slide

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PLATE 14.II.—*P. malariae*

1. Young ring form trophozoite of quartan malaria.
2, 3, 4. Young trophozoite forms of the parasite showing gradual increase of chromatin and cytoplasm.
5. Developing ring form trophozoite showing pigment granule.
6. Early band form trophozoite—elongated chromatin, some pigment apparent.
7, 8, 9, 10, 11, 12. Some forms which the developing trophozoite of quartan may take.
13, 14. Mature trophozoites—one a band form.
15, 16, 17, 18, 19. Phases in the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
21. Immature microgametocyte.
22. Immature macrogametocyte.
23. Mature microgametocyte.
24. Mature macrogametocyte. (Courtesy U. S. Public Health Service.)


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is very clean, the skin is cleaned thoroughly before taking the blood, the slide is not allowed to touch the skin, and the blood is dried protected from insects and dust; if utensils used for water and for staining are kept clean, and if the water used in staining and washing is kept free from growth and contamination, these disturbing factors will be eliminated or greatly minimized. Artifacts which may deceive the inexperienced will be found frequently to lie above the blood plane, or they may be refractile and focus out of the field unevenly. Sometimes red dots appear in the preparation without any visible cytoplasm. It is possible that some of these are at times remnants of malaria parasites, but *one should never call a slide positive on these dots alone*. The dots may be staphylococci or other cocci or they may be products of degeneration of the red cells. If the dots are evenly distributed throughout the smear they are more likely to be associated with the cells, than if they are found in clumps or only in a part of the smear. If a red stained coccus from the skin or small nuclear remnant lies adjacent to a blue particle of cellular substance or fibrin, there may be a resemblance to a parasite. This rarely occurs more than once or twice on a single slide and numbers of free cocci will usually be found also, whereas in well-stained smears free chromatin dots are rarely found. As one becomes more familiar with the thick film one is less likely to mistake any foreign body for a parasite. According to Barber and Komp a good general rule is not to consider anything a parasite which may be interpreted as an artifact. Thick films should not be diagnosed as positive on what appears to be only one parasite. The parasite should be unmistakable or search should be continued until others are found. If this is impossible additional smears should be made."

This Bulletin (see Bibliography) should be studied carefully by all students and technicians who wish to become expert in the identification of malaria parasites.

PLATE 14.III.—*P. falciparum*

1. Very young ring form trophozoite.
2. Double infection of single cell with young trophozoites, one a "marginal form", the other "signet ring" form.
3, 4. Young trophozoites showing double chromatin dots.
5, 6, 7. Developing trophozoite forms.
8. Three medium trophozoites in one cell.
9. Trophozoite showing pigment, in a cell containing Maurer's spots.
10, 11. Two trophozoites in each of two cells, showing variation of forms which parasites may assume.
12. Almost mature trophozoite showing haze of pigment throughout cytoplasm. Maurer's spots in the cell.
13. Aestive-autumnal "slender forms".
14. Mature trophozoite, showing clumped pigment.
15. Parasite in the process of initial chromatin division.
16, 17, 18, 19. Various phases of the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
21, 22, 23, 24. Successive forms in the development of the gametocyte—usually not found in the peripheral circulation.
25. Immature macrogametocyte.
26. Mature macrogametocyte.
27. Immature microgametocyte.
28. Mature microgametocyte. (Courtesy U. S. Public Health Service.)

TABLE 14.10

Differentiation of the Three Common Species of Plasmodia

	P. vivax	*P. malariae*	*P. falciparum*
Asexual forms in blood film	All stages	All stages	Rings only (very rarely other stages)
Ring forms	Indistinguishable. $\frac{1}{3}$ diam. of r.b.c.	Usually larger	Usually smaller. $\frac{1}{6}$ diam. of r.b.c. More often have 2 chromatin dots, often at opposite poles. More often at edge of red cell. More often several rings in one red cell.
Larger trophozoites	Irregular in shape (ameboid) with vacuole	Oval or band forms, no vacuole	Round or oval (usually not seen in blood film)
Mature schizont	Larger than a normal r.b.c.	Slightly smaller than a normal r.b.c.	Distinctly smaller than a normal r.b.c.
Number of merozoites	16–24	16–12 (8)	8–32
Gametocytes	Round or oval	Round or oval	Banana-shaped
Pigment	Fine, golden brown	Coarse, dark brown	Coarse, very dark brown
Alterations in r.b.c. after ring stage:			
Size	Enlarged	Not enlarged. May be contracted	Unaltered
Color	Pale	Not pale; may be copper colored	Pale
Granules	Fine, "Schuffner's dots"	None	Coarse, "Maurer's dots"

Pigmented Leukocytes. The hemozoin pigment liberated into the blood stream when schizogony is completed is taken up mainly by the large mononuclear phagocytes of the body, including the monocytes of the blood. In heavily infected cases the pigment or pigmented parasites may be found also in the polymorphonuclear leukocytes. Pigmented leukocytes are practically diagnostic of heavy malaria infections.

If they are found in the absence of parasites the infection is probably *P. falciparum*, and many ring forms will probably be found on the following day. No diagnosis on the basis of pigment alone can be made.

Blood Platelets. Blood platelets are sometimes mistaken for malaria parasites by inexperienced observers when they lie on red blood cells. Large platelets may be the same size and shape as the crescents of *P. falciparum*. Such mistakes can be avoided by comparing suspected objects with other platelets in the same preparation.

Interpretation

Malaria must be kept in mind in every patient who has lived in or visited an endemic region. It must also be kept in mind in regions where it is not known to be endemic, since modern habits of travel may initiate sporadic local epidemics. Malaria is not infrequently transmitted by blood transfusion; *P. malariae* especially may remain latent in man for as long as 30 years and yet give rise to a new clinical infection following a transfusion from an unsuspected latent case. Epidemics of malaria also have occurred among drug addicts employing a common syringe for intravenous drug injection.

Inability to find parasites in an ill person does not eliminate malaria as the cause of the illness. In the first attack of malaria the parasites are usually numerous, but in relapses they may be difficult to find. Parasites may be absent from the blood film one day and present the next, especially in falciparum malaria. Examinations on consecutive days are therefore imperative. In subclinical cases one examination even by the thick film method will reveal only about fifty per cent of the infections. The therapeutic test is justified if symptoms point strongly to malaria.

When malarial parasites are rare in the blood they may be most difficult to demonstrate on a thin blood film. Since a good thick blood film concentrates the blood about 20 times, the great advantage of thick blood films when parasites are rare is obvious. However, it requires special training to enable a laboratory worker to identify malarial parasites in thick film preparations. It is folly to expect any laboratory worker without such special training and skill to render this service. Every laboratory should report its positive malaria films by identifying the species if possible and giving some indication of the stages of the parasites present. The actual enumeration of parasites present is seldom of any significance except in *P. falciparum* infections, for quartan parasitemias seldom exceed 10,000 per ml. and tertian rarely over

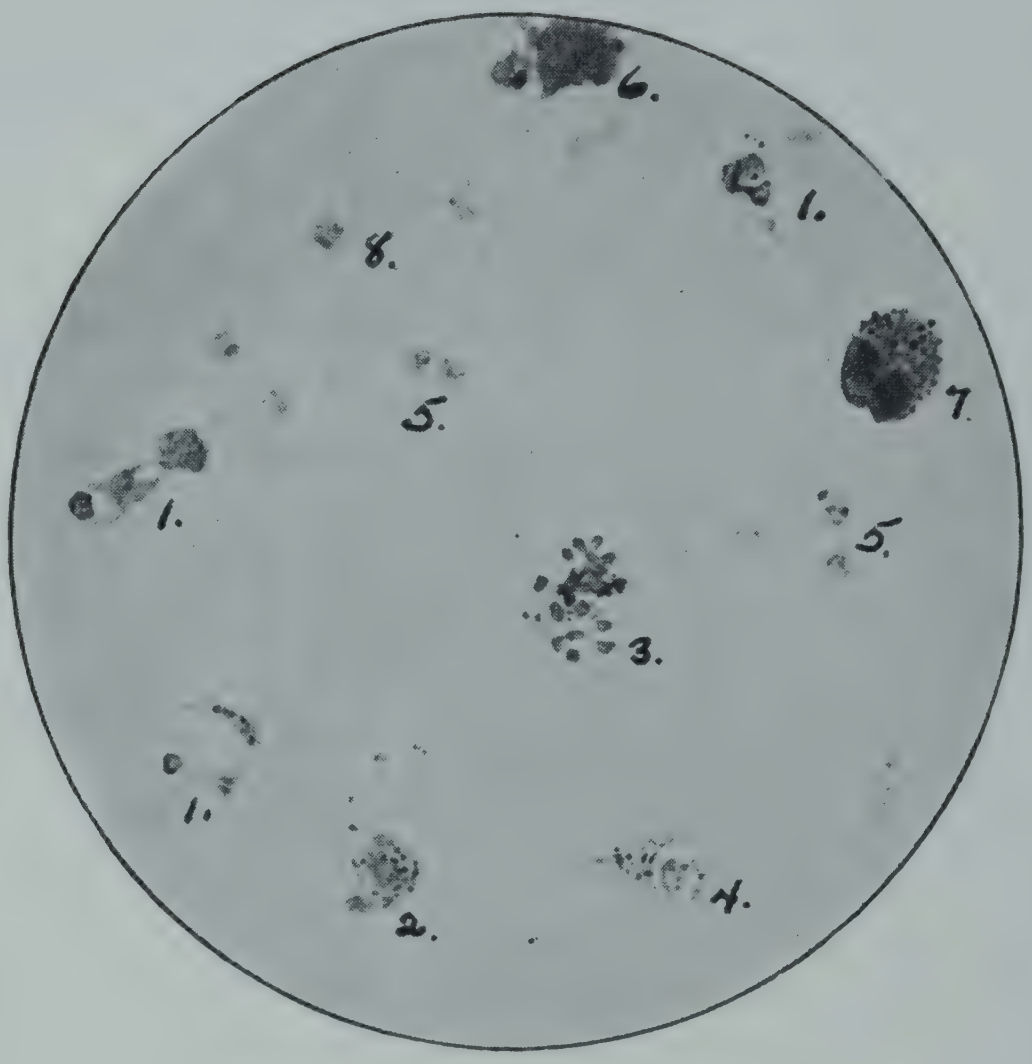
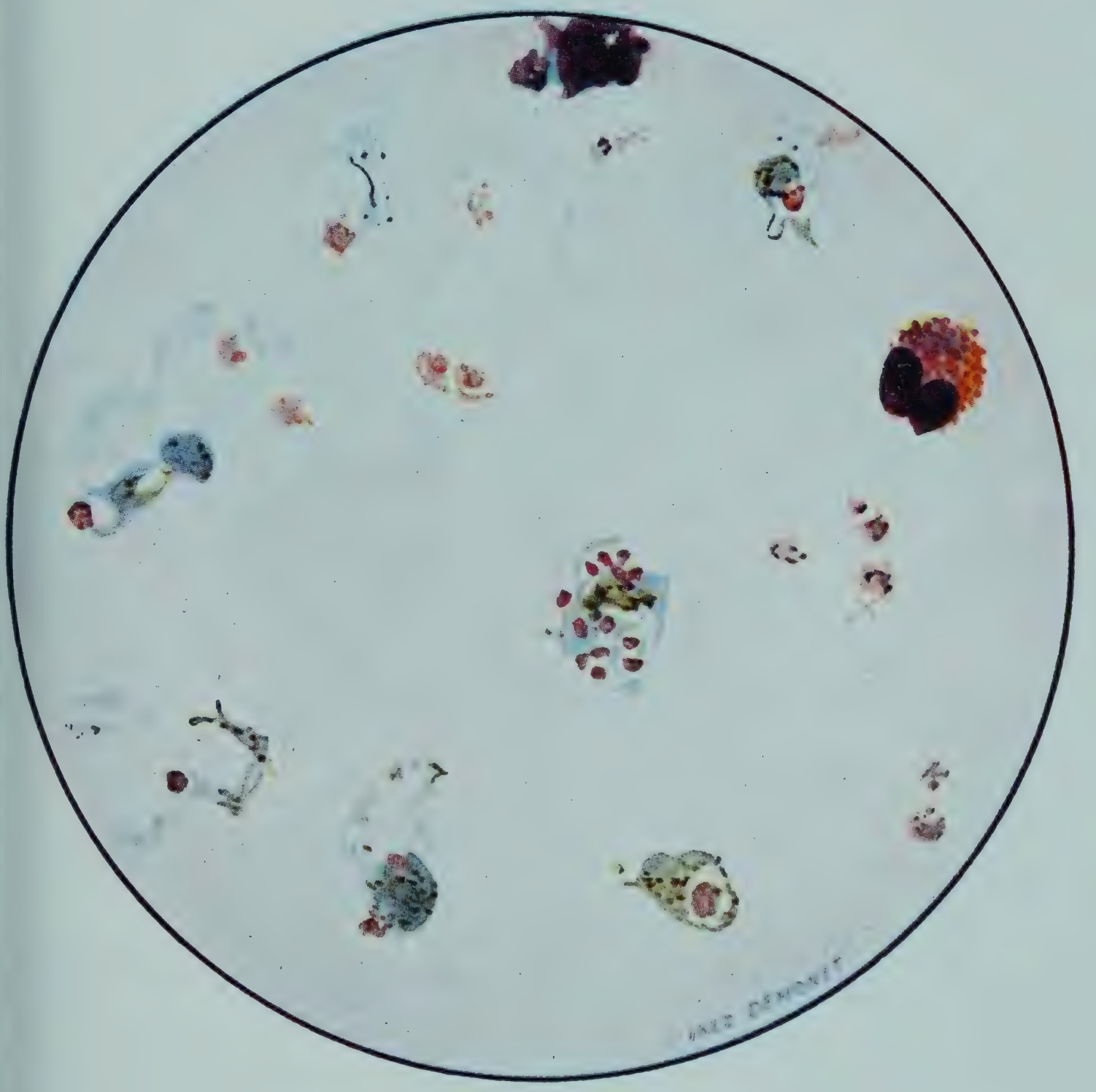


PLATE 14.IV.—*P. vivax*—thick film

1. Amoeboid trophozoites.
2. Schizont—2 divisions of chromatin.
3. Mature schizont.
4. Microgametocyte.
5. Blood platelets.
6. Nucleus of neutrophile.
7. Eosinophile.
8. Blood platelet associated with cellular remains of young erythrocytes.

(Courtesy U. S. Public Health Service.)



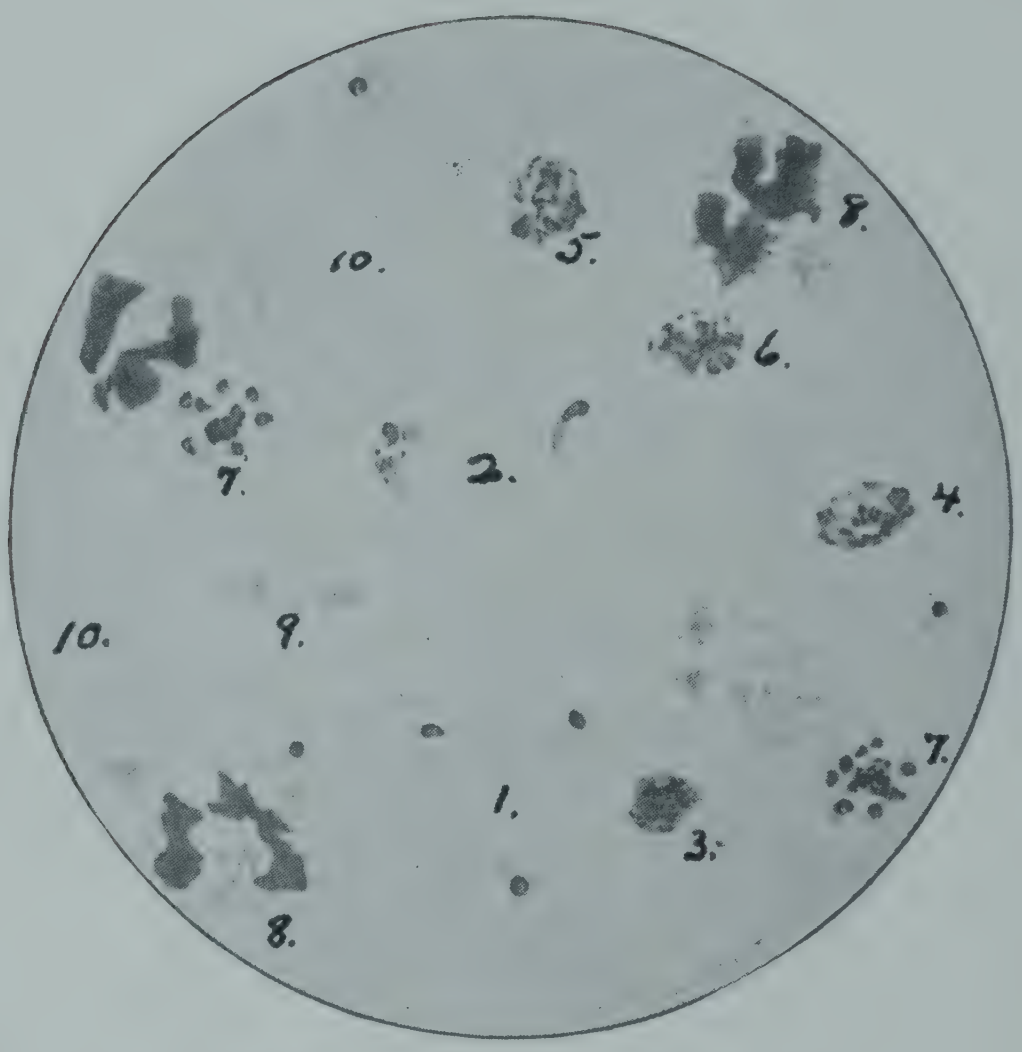
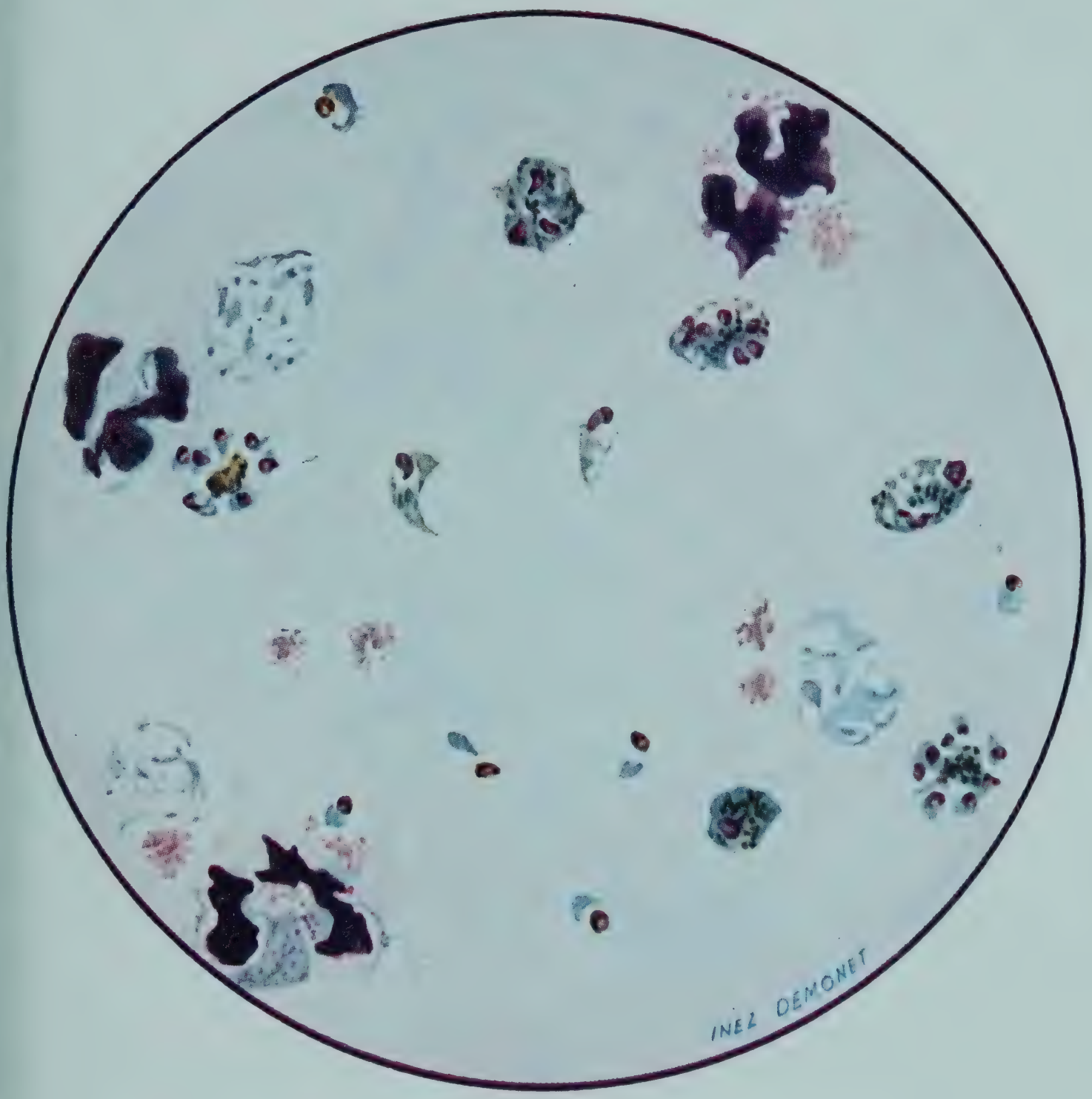


PLATE 14.V.—*P. malariae*—thick film

1. Small trophozoites.
2. Growing trophozoites.
3. Mature trophozoites.
4, 5, 6. Schizonts (presegmenting) with varying numbers of divisions of the chromatin.
7. Mature schizonts.
8. Nucleus of leukocyte.
9. Blood platelets.
10. Cellular remains of young erythrocytes. (Courtesy U. S. Public Health Service.)


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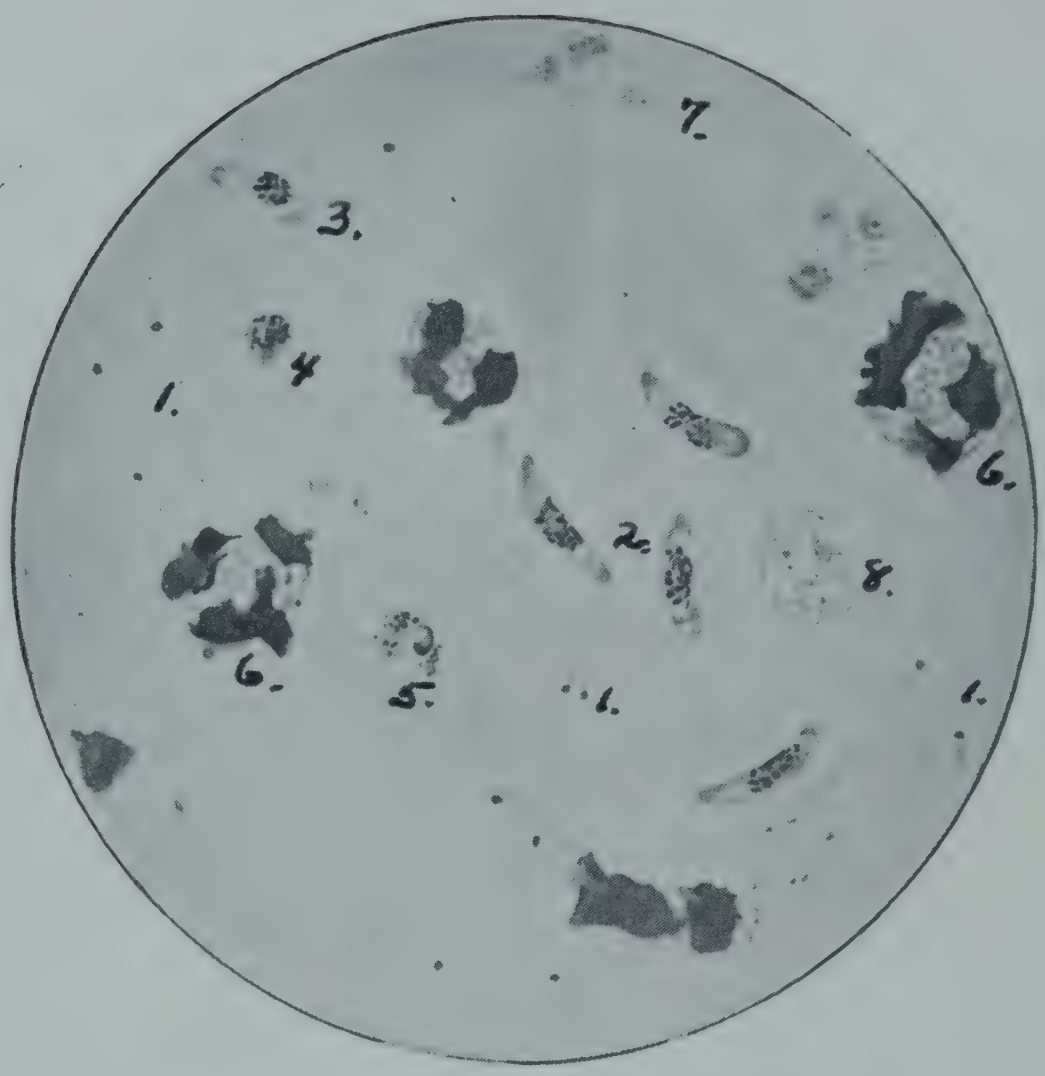
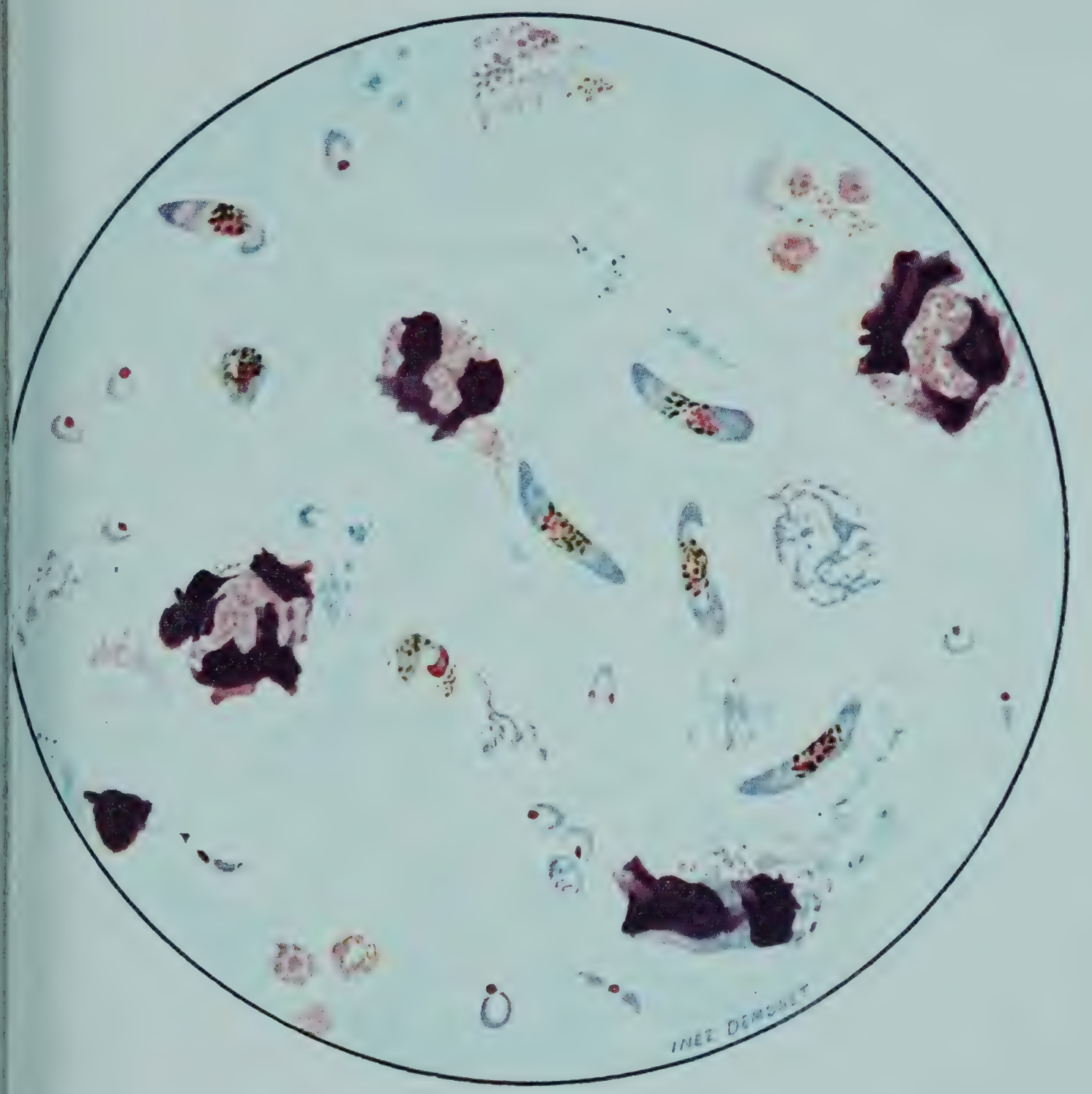


PLATE 14.VI.—*P. falciparum*—thick film

1. Small trophozoites.
2. Gametocytes—normal.
3. Slightly distorted gametocyte
4. "Rounded-up" gametocyte.
5. Disintegrated gametocyte.
6. Nucleus of leukocyte.
7. Blood platelets
8. Cellular remains of young erythrocyte. (Courtesy U. S. Public Health Service.)


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50,000 per ml. However, *P. falciparum* infection has no usual limits of parasitemia and since coma and deaths occur frequently from the obstruction of fine capillaries in the brain by large numbers of these parasites, an enumeration may be of considerable significance, especially in following an acute ill case.

The Helminths (Worms)

There are two phyla of medically important helminths, the Platyhelminthes (flatworms) and the Nemathelminthes (roundworms).

The Platyhelminthes (flatworms)

Two classes are of medical importance, the Cestoda, ribbon or tapeworms, and the Trematodes, flattened, oval, or leaf-shaped worms frequently termed the flukes.

THE CESTODA (TAPEWORMS)

There are six flat tape-like worms that commonly inhabit the intestinal tract of man. In addition, a seventh tapeworm, *Echinococcus granulosus*, is of medical importance, for man serves as the intermediate host, with severe systemic infection occurring due to the larvae which develop into hydatid cysts. Tapeworms are a series of individual units with one head which is the mother segment. It is smaller than the daughter segments that arise from the head by proliferation and which are called the proglottids. The head therefore is the portion of the worm of primary significance and must be expelled to eradicate the infection; otherwise the segments reform from the retained head. The nervous system originates in the head, which has one or more organs of attachment—the scolex, equipped with grooves or suckers and frequently hooklets. The scolex enables the parasite to fix itself securely to the intestinal wall. There is no digestive tract, food being absorbed directly from the host's intestinal tract. Posterior to the head is a long slender neck, the growing portion. Here the proglottids develop from the posterior portion of the neck. First, the anterior undifferentiated segments or immature proglottids form, which develop progressively to the more posterior mature segments. The mature proglottids each contain both the male and female reproductive organs; the gravid uterus largely fills the interior of the proglottid. The eggs are liberated through one or two genital pores, depending on the species. New segments are being continually formed and the older gravid segments gradually break off. The number of segments varies from a few to a few thousand.

TABLE 14.11

Seven Tapeworm Infections of Man

Genus and Species	Disease	Definitive Host	Intermediate Host	Infection Acquired
Diphyllobothrium latum	Diphyllobothriasis (fish tapeworm)	*Man*	Copepods, fish	Ingestion of raw or poorly cooked fish
Dipylidium caninum	Dipylidiasis (dog tapeworm)	*Man*, dog	Fleas and lice	Ingestion of infected fleas or lice
Hymenolepis nana	Dwarf tapeworm infection	*Man*, rats, and mice	Man, rats, and mice	Ingestion of ova
Hymenolepis diminuta	Rat tapeworm infection	*Man*, rats, and mice	Rat fleas and other insects	Ingestion of infected fleas
Taenia saginata	Taeniasis (beef tapeworm)	*Man*	Cattle	Ingestion of poorly cooked infected beef
Taenia solium	Taeniasis (pork tapeworm)	*Man*	Hog, man	Ingestion of poorly cooked infected pork
Echinococcus granulosus	Echinococcosis (Hydatid disease)	Dogs and other canines	Man, herbivorous animals	Ingestion of ova

The usual site of attachment is in the ilium. Table 14.11 shows the seven medically important tapeworm infections.

Life Cycle

Table 14.11 indicates that man is the definitive host in five of these seven infections and the intermediate host in one, Echinococcus, where dogs serve as the definitive host. *Taenia solium* ova may also infect man, developing into the larval form, causing cysticercosis. *Hymenolepis nana* requires no intermediate host; man serves as host for both adults and larvae. Otherwise, the ova of these parasites, when ingested by an appropriate intermediate host, develop the larval forms enclosed in a cyst bladder-like structure, the cysticercus, or lodged in the muscle tissue. Man becomes infected by ingestion of this infected meat when insufficiently cooked.

Diphyllobothrium latum (*Broad or Fish Tapeworm*)

The adult inhabits the small intestine of man, dog, cat, bear, or other fish-eating mammal and measures up to 10 meters in length with 3,000 or more segments. The head is spatula-shaped and has two slitlike suckers, but no hooklets (see Table 14.12). The eggs are liberated from the gravid segments through a birth pore without rupture of the segments. After 2 to 5 weeks of development in water the embryo escapes through the opercular opening of the egg. The ciliated embryo swims about in the water and if swallowed by certain small crustaceans

(Cyclops or Diaptomus) it penetrates into the body cavity and develops into a procercoid larva. If the crustacean is swallowed by a fresh-water fish (pike, perch, trout, etc.) it escapes in the intestine, penetrates into the body cavity and develops in the muscles of the fish into a plerocercoid. This is a white, flat, wrinkled, unsegmented object without definite anatomical characteristics except for two suckers at the anterior end and measures one to several centimeters in length. Man and other animals become infected by eating infected insufficiently cooked fish.

The egg (see Fig. 14.10 [fig. 13] and Table 14.13) is broad oval, one end slightly smaller with an operculum and the other end slightly thickened. The average size is 70 by 45 micra. The shell is thin but with double contour, sometimes light brown in color. The embryo is surrounded by a granular yoke mass nearly filling the shell. It has no hooklets until fully developed several weeks after leaving the intestine of the host. There are usually enormous numbers of these eggs in the stools of patients harboring even a single adult worm, since each gravid segment has a birth pore and eggs are constantly being discharged into the intestinal tract.

Dipylidium caninum (*Dog Tapeworm*)

This common dog parasite infects about 50 per cent of dogs in the United States; occasionally it infects man, but usually only a single adult worm is present. The adult worms are generally 20 to 40 cm. in length, consisting of scolex, slender neck, and 60 to 175 proglottids 2.5 to 3 mm. in width (see Table 14.12). The scolex has four prominent

TABLE 14.12

Differential Characteristics of Intestinal Tapeworms

Parasite	Scolex Suckers	Hooks	Length of Worm	Number of Proglottids	Gravid Uterus
			cm.		
Diphyllobothrium latum	2	No	300–1,000	3,000–4,000	Central coiled
Dipylidium caninum	4	Yes	15–70	15–170	Polygonal egg sac
Hymenolepis nana	4	Yes	0.5–4.5	100–200	Irregular sac-like
Hymenolepis diminuta	4	No	10–60	800–1,000	Irregular sac-like
Taenia saginata	4	No	400–800	1,000–2,000	15–30 lateral branches
Taenia solium	4	Yes	200–700	800–1,000	7–13 lateral branches

oval suckers and several rows of thorn-shaped hooklets. The infection in man (mostly children) occurs from the ingestion of infected dog fleas or dog lice. However, symptoms are rare, if any.

The ova can rarely be demonstrated in feces because they are not liberated from the gravid proglottids within the host. Rather, the intact proglottids are passed and disintegrate outside the body of the host (see Table 14.13).

Hymenolepis nana (*Dwarf Tapeworm*)

The adult worm measures 25 to 40 mm. in length and has up to 200 segments. The head has 4 round suckers and 1 row of hooklets (see Table 14.12). The worm inhabits the small intestine and the eggs are liberated by rupture of the gravid segments in the intestine of the host. The embryo may hatch from the egg in the intestine and may penetrate into the mucosa and develop into the cysticercoid larva. After a period of incubation the larva escapes into the lumen of the intestine and develops into an adult. Eggs which are passed in the feces may be swallowed by man, in which case the embryo hatches in the small intestine, penetrates into the mucosa and develops as just described. This life cycle may also occur in rats and mice. The egg (Fig. 14.10 [fig. 11]) is round or slightly oval, and has an average diameter of 40 micra. The outer shell appears as a thin, smooth, single line. Inside this is a clear space containing granules and thread-like filaments. Within this space is the inner shell, about half the diameter of the outer shell and similar in thickness. The embryo, which is often separated slightly from the inner shell by a clear space, is granular and contains three pairs of hooklets (see Table 14.13).

Hymenolepis diminuta (*Rat Tapeworm*)

This is occasionally found in man. The adult worm inhabits the small intestine. It measures 20 to 60 cm. in length, being much larger than *H. nana*. The head has 4 round suckers but no crown of hooklets (see Table 14.12). The eggs are liberated by rupture of the gravid segments in the intestine. The intermediate hosts are various insects, especially rat fleas. The embryo hatches in the gut of the insect, penetrates into the body cavity and becomes a cysticercoid larva. Man and the rat become infected by swallowing such infected insects.

The egg (Fig. 14.10 [fig. 12]) differs from that of *H. nana* in being larger (average diameter 70 micra), brown in color, and in having a thick,

dark brown, radially striated outer shell and a clear intermediate space (see Table 14.13). The embryo usually fills entirely the space inside the thin inner shell. The three pairs of hooks can be seen in the embryo.

Taenia saginata (Beef Tapeworm) and Taenia solium (Pork Tapeworm)

Taenia saginata. This infection is common is the United States. The adult worm averages 10 to 15 meters in length and has up to 2,000 segments (see Table 14.12). The larval state, *Cysticercus bovis*, exists in cattle, measuring about 4 to 10 mm. in diameter, and is white in color. The scolex possesses four suckers but no hooklets.

Taenia solium. This infection is very rare in the United States. The adult worm averages 2 to 8 meters in length and has up to 900 segments (see Table 14.12). The larval stage, *Cysticercus zellulosae*, exists in hogs and man. It is 5 to 10 mm. in diameter and white in color. The scolex possesses 4 suckers and 2 rows of hooklets. The adult stages of both of these worms inhabit the small intestine of man. The eggs are liberated from the gravid segments by rupture of the segments. This may occur in the intestine or after the segments have been swallowed by the intermediate host. The embryo escapes from the egg in the intestine of the intermediate host, penetrates into blood vessels and is carried to the striated muscles where it develops into a cysticercus. Infection of man occurs by eating insufficiently cooked beef (*T. saginata*) or pork (*T. solium*).

The eggs of the two species are practically indistinguishable (Fig. 14.10 [figs. 9 and 10]). They are round or slightly oval, with a diameter of 30 to 35 micra. The color is yellow or brown and the shell very thick and radially striated (see Table 14.13). The space within the shell is entirely filled by the embryo which contains three pairs of hooklets about half the diameter of the embryo in length, although frequently these are difficult to see. At the time the egg is liberated from the tapeworm by disintegration of a segment, it is enclosed within an outer clear, embryonic membrane about twice the diameter of the inner shell and containing a number of granules and vacuoles (Fig. 14.10 [fig. 9]). This membrane has usually disappeared by the time the egg is found in the stool. Eggs of Taenia may not be found in the stools although the patient harbors the parasite, because the only method by which the eggs can escape from the uterus of a segment is by rupture or disintegration of the segment, and it more often happens that the segments are passed intact in the stool. The patient often makes the diagnosis of Taenia infection himself, and should be instructed to bring to the laboratory for identification any segments which he passes.

TABLE 14.13
Differential Characteristics of Intestinal Tapeworm Ova

Genus and Species	Size	Shape	Operculaed	Features
	μ			
Diphyllobothrium latum	45 x 70	Oval	Yes	Immature, no hooks
Dipylidium caninum	36 x 40	Globular	No	In packets of 10–12 embryos, 6 hooks each
Hymenolepis nana	37 x 47	Globular	No	Embryo, 6 hooks, double membrane
Hymenolepis diminuta	65 x 72	Oval	No	Embryo, 6 hooks
Taenia saginata	25 x 35	Oval	No	Thick brown, radially striated, 6 hooks
Taenia solium	35	Round	No	Thick brown, radially striated, 6 hooks

It is important to make a differential diagnosis between *T. saginata* and *T. solium*. *T. saginata* is important because of its parasitic inhabitation in the intestine, although usually not giving rise to any symptoms. *T. solium*, on the other hand, is a very dangerous parasite to man because if the eggs of this worm are swallowed by man he may act as the intermediate host. The larvae liberated from the eggs may be carried to any part of the body, where they encyst, developing into *Cysticercus cellulosae*. The brain is an especially favorable place for their development, and here they cause severe symptoms and death.

The simplest method of differentiating *T. saginata* from *T. solium* is by injecting a little India ink into the genital pore on the lateral margin of a gravid segment, using a 26 gauge needle on a hypodermic syringe. The ink spreads into the branches of the uterus, after which pressing the segment between two glass slides makes the uterus easily visible. The gravid uterus of *T. saginata* has more than 15 lateral branches, while that of *T. solium* has from 7 to 13 (see Fig. 14.7).

Examination for Tapeworm Heads

It is very important to obtain and identify the head (scolex) of tapeworms such as *Taenia saginata*, *Taenia solium*, and *Diphyllobothrium latum* after treatment.

If the head is not obtained, the physician and the patient must remain in suspense for weeks waiting for the reappearance of segments or ova in the feces Treatment should be preceded by the avoidance of solid food for 24 hours and by thorough purging with salts. Administration of the anthelmintic by duodenal tube should be advocated. This causes release of the head in practically all cases,

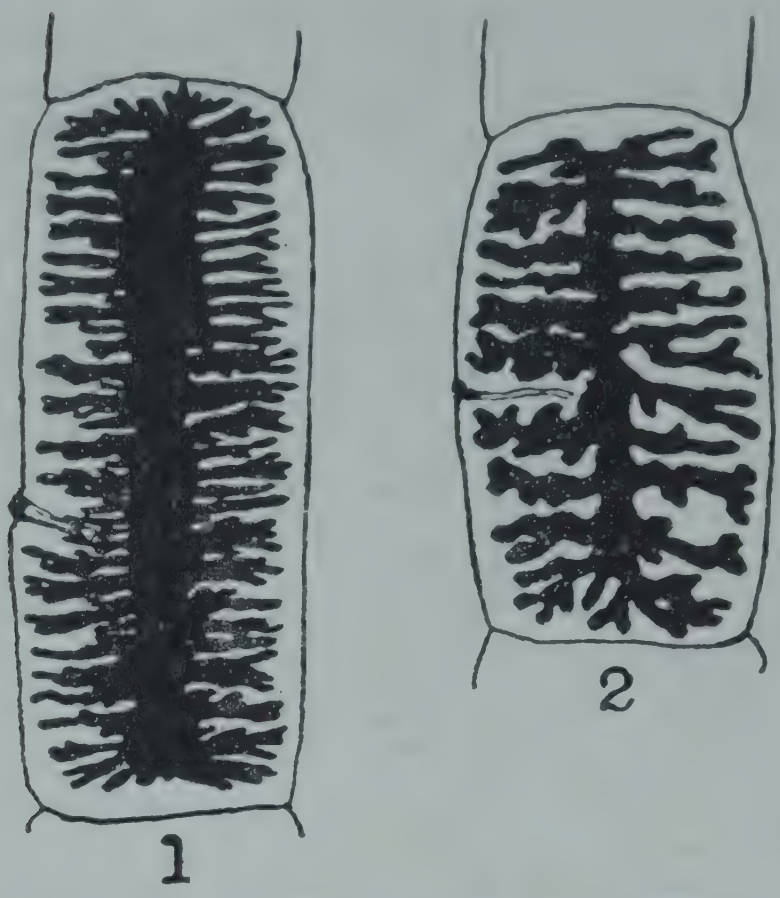


FIG. 14.7. Gravid segments of (1) *Taenia saginata* and (2) *Taenia solium*. Note that the uterus of *T. saginata* has more than 14 primary lateral branches, whereas that of *T. solium* has less than 12. From E. C. Faust, Human Helminthology, Lea and Febiger, 1929.

whereas administration by mouth frequently fails to release the head. The anthelmintic should be followed in 15 minutes by a large dose of salts administered through the duodenal tube. The resulting evacuation should contain practically no fecal material if the patient has been properly prepared. It should be collected in a bedpan or vessel containing a small amount of warm water. Toilet paper should not be put into the container. The entire specimen should then be taken to the laboratory without transfer to another container if possible. If transferred, the original container should be thoroughly rinsed and the rinsings added to the specimen. Subsequent evacuations should be collected in the same manner and saved for examination if necessary. The head is usually found in the same evacuation as the main portion of the worm, most often the first evacuation. It may be attached to the body of the worm or may have broken off, in which case it may be only a centimeter or so in length. Since the head of a Taenia is only 1 to 2 mm. in diameter it easily may be overlooked.

The evacuation should be treated in one of two ways. (1) It may be strained through a 20-mesh wire sieve, followed by rinsings from the original container and tap water to wash through the fine debris. Then the sieve should be inverted in a black-bottomed pan (Fig. 14.8) or a large glass dish and rinsed thoroughly with water. (2) The evacuation may be poured into a large beaker, followed by rinsings from the original container, and washed until clear by careful, repeated sedimentation and decantation. The sediment and rinsings from the beaker should then be poured into the black pan or glass dish. The head, if present, can be found against a black background by a hand lens and identified under the microscope.



FIG. 14.8. Showing a satisfactory apparatus for the recovery of the tape-worm head. It consists of an enamel pan which is painted black with asphalt paint. The sieve is used to strain the specimen; the wooden applicator points to the head. From Magath and Brown, Jour. Amer. Med. Assn. 88: May 14, 1927.

The heads of the three large tapeworms can be differentiated as follows: *T. saginata* has four round suckers but no crown of hooklets; *T. solium* has four round suckers and two rows of hooklets; *D. latum* has a slender spatulate-shaped head with two longitudinal slit-like suckers and no crown of hooklets.

Echinococcus granulosus (*Echinococcosis, Hydatid Disease*)

The livestock-raising areas of the world have the heaviest infection, with dogs, wolves, etc., serving as the definitive hosts, while man, sheep, cattle, and horses are the common intermediate hosts.

Life cycle. Ova evacuated in the feces of the definitive host are ingested by man, the embryos are liberated in the duodenum, pass through the intestinal wall and are carried by the blood to various parts of the body. Most frequently they lodge in the liver via the portal stream. Here, if not phagocytized, they develop into hydatid cysts reaching up to 7 cm. in diameter. The common unilocular cysts have an inner lining studded with brood capsules which rupture, liberating many scolices into the yellowish fluid. The symptoms in man depend upon the number

and location of these cysts. The symptoms are those of a slow-growing tumor in the area of location. X-ray plates frequently show these round cysts filled with fluid.

Identification of hydatid cysts. A positive diagnosis usually is made in the laboratory by examination of the fluid. Hydatid cyst fluid is pale yellow, pH 6.7 to 7.2, with a specific gravity of 1.012. Thousands of scolices may be present in the fluid as well as daughter cysts and brood capsules. Quite reliable antigens have been developed for serologic and skin tests. Belding states that "If the eosinophilia exceeds 5 per cent and the intracutaneous and alexin-fixation reactions are positive, diagnosis is fairly definite." Diagnostic aspiration of these cysts is counter-indicated because of leakage and the resulting secondary echinococcosis.

It is of special interest to note that when man serves as the accidental intermediate host of an intestinal tapeworm, severe destructive pathology results. Ingestion of the mature eggs of *Taenia solium* or *Echinococcus granulosus* therefore results in severe infection for which we have as yet no effective chemotherapy, and surgery offers the only hope of eradication. In such cases great care must be exercised in handling these cysts in order not to contaminate the tissue with cyst fluid.

THE TREMATODES (FLUKES)

The trematodes are the second class of flatworms infecting man. These parasitic worms, called flukes, all require as an intermediate host a mollusc. Therefore, human cases occur only in regions where the appropriate snails exist. Native infections are rare in the United States, although some of the intermediate snail hosts do exist here. The increasing numbers of fluke infections predominantly are acquired abroad.

Morphology

The flukes are flat, often leaf-like, unsegmented small worms, and all except the schistosomes are hermaphroditic with operculated ova. From the ova are liberated larvae, the active ciliated miracidia which require specific species of snails which they penetrate and in which they reproduce in large numbers. These give rise to a free-living, often motile form such as the cercaria of schistosomes. These cercariae directly penetrate the skin of man and start their development to adult worms with man as their definitive host; other species gain entry by being ingested in an encysted form.

The more important human fluke infections are grouped most conveniently by their site of habitat. Thus, there are three blood flukes,

TABLE 14.14

The Eight Major Trematode (Fluke) Infections of Man

Fluke	Disease	Adult Habitat	Endemic Areas	Infection Acquired
Blood				
Schistosoma haematobium	Schistosomiasis, bilharziasis	Blood, veins of bladder	Widespread in Africa	Skin, contact with infested water
Schistosoma mansoni	Schistosomiasis, schistosomal dysentery	Blood, veins of colon	Africa, West Indies, S. America	Skin, contact with infested water
Schistosoma japonicum	Schistosomiasis, katayama disease	Blood, veins of colon	Japan, China, Philippines	Skin, contact with infested water
Liver				
Fasciola hepatica	Fascioliasis, liver rot	Liver, bile ducts	Sheep-raising areas of the world	Ingestion of infested water or uncooked water plants
Clonorchis sinensis	Clonorchiasis	Liver, bile ducts	Orient	Ingestion of uncooked infected fish
Opisthorchis felineus	Opisthorchiasis	Liver, bile ducts	Eastern Europe, Siberia	Ingestion of uncooked infected fish
Intestine				
Fasciolopsis buski	Fasciolopsiasis	Intestinal duodenum	Orient, Bengal	Ingestion of infected water plants
Lung				
Paragonimus westermani	Paragonimiasis	Lung	Orient, Pacific islands, S. America	Ingestion of uncooked infected crustaceans

three liver flukes, one intestinal fluke, and one lung fluke of major significance (see Table 14.14).

The Blood Flukes (Schistosoma haematobium, Schistosoma mansoni, and Schistosoma japonicum)

General considerations. The adult males and females of these trematodes live together in copulation. *Schistosoma haematobium* inhabits principally the veins of the urinary bladder, rarely those of the intestine. *Schistosoma mansoni* inhabits principally the veins of the rectum and lower colon, rarely those of the urinary bladder. *Schistosoma japonicum* inhabits principally the veins of the colon, never those of the urinary bladder. The eggs of all three worms are deposited in capillary tributaries to these veins or at times liberated in the venous blood stream. The embryo within the egg secretes a toxic material which penetrates

through the egg shell, the capillary wall, and causes the development of a minute abscess in the surrounding tissue. From the mucosal wall of the intestine or bladder the abscess may rupture into the lumen, liberating the egg with blood and pus in the feces or urine. If the egg is liberated into the blood stream and carried to the liver or lungs, the abscess results in fibrosis in these organs. The eggs which escape in feces or urine hatch quickly in water and liberate the ciliated embryo which penetrates into a suitable snail intermediate host. The embryo develops into two generations of sporocysts and within the second-generation sporocysts develop the infective, fork-tailed larvae or cercariae. These escape from the snail, are actively motile, and penetrate the skin of man or other mammals contacted in the water. They migrate into the venous circulation and are carried through the lungs and general circulation to the veins of predilection in which they develop into adults.

Identification of ova. The eggs of the schistosomes have thin shells with a single contour narrower at the anterior end. Within the shell there is usually a fully developed ciliated embryo, the *miracidium*. The miracidium may be facing either end of the shell. Between miracidium and shell there may be granules and globules. These eggs are sticky and accumulate fecal debris about them. The shells are fragile and if examined in tap water may burst during the examination, liberating the miracidium which swims about actively. For this reason normal saline must be used in any concentration method. The egg of *S. haematobium* (Fig. 14.10 [fig. 14]) is long, oval, and sharply pointed at one end (terminal spine). Average size is 150 by 50 micra. These eggs are usually found in urine, rarely in feces. The centrifuged urine sediment may contain many ova with hatching taking place, yielding a free swimming miracidium. The endemic region is Africa. The egg of *S. mansoni* (Fig. 14.10 [fig. 15]) is long and oval, with a prominent lateral spine; the average size is 140 by 65 micra. These eggs are usually found in feces, rarely in urine, and are never very numerous. The endemic regions are Africa, South America, and the West Indies. The egg of *S. japonicum* (Fig. 14.10 [fig. 16]) is broadly oval and has a small knob located antero-laterally, which is usually invisible because of the debris sticking to the egg. The average size is 85 by 60 micra and it is found only in the stools. Often these eggs are quite numerous. The endemic regions are Central and South China, Indo-China, Philippines, Formosa, Japan, and the Celebes (see Table 14.15).

In view of the fact that many hundreds of cases of *Schistosomiasis japonica* occurred among our military forces in the Philippines in World

War II, and since light infections going undiagnosed may ultimately develop symptoms, laboratories should be prepared to use the most accurate technique for detecting the very few eggs of Schistosoma usually present in the feces. The following procedure is the most practical for general use, is probably as accurate as any other, and permits hatching of eggs for the detection of miracidia if the infection is so light that the eggs cannot be found.

Sedimentation. The entire stool specimen must be used, as eggs may be most unevenly distributed in it. Emulsify the specimen in a small amount of normal saline solution, 0.8 per cent NaCl in water. This solution prevents hatching during the procedure of sedimentation. Mix the stool emulsion in 500 cc. of this solution and filter this suspension through a 20-mesh wire sieve in a funnel, into a 2-liter glass cylinder, washing the residue in sieve with saline until the cylinder is filled. Allow the suspended material to sediment for one hour. Then siphon off the supernatant, replacing it with fresh saline, and repeat until the supernatant is clear. The sedimentation time may be reduced as the supernatant becomes clearer. After siphoning off the final supernatant examine small samples from the bottom of the sediment for eggs (see Table 14.15).

Hatching. If eggs are not found, or if it is desired to observe miracidia, transfer the sediment to a 2-liter Erlenmeyer flask and fill flask into neck with chlorine-free tap water or distilled water. The pH of the water should be 7.6 to produce maximum hatching. Allow to stand at room temperature and examine the supernatant at frequent intervals for miracidia. *S. japonicum* miracidia will be found most frequently near the surface pellicle. Those of *S. mansoni* are more evenly distributed in the water. The miracidia can be seen with a hand lens or dissecting microscope directed horizontally, with cross illumination, against a dark background. They swim swiftly, changing direction when they meet an obstruction. They must not be confused with other free-living organisms in water. If numerous they can be pipetted off and identified under higher power. Successive crops of miracidia may be hatched by pouring off supernatant daily and replacing it with fresh water at pH 7.6.

The Liver Fluke (Fasciola hepatica, Clonorchis sinensis, and Opisthorchis felineus

These three liver flukes inhabit the bile ducts, particularly those of the left lobe of the liver. A few worms cause few or no symptoms but large numbers cause liver destruction, inflammation, and fibrosis. However, rarely if ever do they cause complete obstruction of the bile ducts. The laboratory identification of either the adult worm or the ova establishes the presence of that specific infection (see Table 14.15 for characteristics of ova).

Fasciola hepatica. This fluke is prevalent in all sheep-raising countries, including the United States. The adult worm is rather conical or

leaf-shaped—like a modern candle-shaped electric bulb in outline. It is 30 by 13 mm., and quite flat. The ova are passed immature about 80 by 140 microns with an indistinct operculum. The granular protoplasmic structure of the developing embryo is quite uniform (see Table 14.15 for characteristics of ova). Man acquires the infection by ingestion of raw green vegetables containing the encysted metacercaria.

Clonorchis sinensis. This fluke infection is apparently confined to Japan, Korea, China, and Indo-China. It is a hermaphroditic trematode inhabiting the bile ducts and is flat and long-oval in shape, and measures 10 to 25 mm. in length but only 3 to 5 mm. in width. The eggs are liberated from the uterus of the worm into the bile ducts, carried to the intestine, and passed in the feces. The embryo hatches only after the egg is swallowed by a suitable snail intermediate host. Several larval generations develop in the snail and the cercariae escape into the water. They encyst under the scales or in the flesh of certain small fresh-water fish. Man and other mammals become infected by eating infected insufficiently cooked fish. The larvae excyst in the duodenum and migrate into the bile ducts. (See Table 14.15 for characteristics of ova.)

Opisthorchis felineus. This fluke is found in eastern Europe, Siberia, Indo-China, Japan, and India. Man is usually an accidental host, for the cat is the common host. The life history is similar to Clonorchia and so will not be repeated. The adult worms measure about 10 by 2 mm. The ova are yellowish-brown, operculated and more slender than Clonorchis with more tapering ends, averaging 12 to 30 microns in length. Man acquires the infection by eating uncooked or inadequately cooked infected fish. (See Table 14.15 for characteristics of ova.)

The Intestinal Fluke, Fasciolopsis buski

This large intestinal fluke producing fasciolopsiasis is a common parasitic infection in the Orient. Its life cycle is similar to *Fasciola hepatica*, with man ecquiring the infection by ingestion of certain water plants raw. These flukes generally attach themselves to the mucosa of the duodenum and jejunum. They are usually rather flat, elongated oval fleshy worms from 20 to 75 mm. in length, 8 to 20 mm. in width, and only 0.5 to 3 mm. in thickness. The eggs are nearly identical with those of *F. hepatica*, indistinctly operculated, and measuring 130 by 80 microns.

Identification. The laboratory identification of all three liver flukes and the intestinal fluke *F. buski* is made by demonstration of the eggs in the feces of infected individuals.

TABLE 14.15

Distinguishing Characteristics of the Eight Trematode (Fluke) Ova

Genus and Species	Size	Operculated	Significant Characteristics
	μ		
Schistosoma hematobium	150 x 60	No	Large terminal spine, active ciliated miracidium
Schistosoma mansoni	155 x 65	No	Large lateral spine, active miracidium
Schistosoma japonicum	60 x 85	No	Little or no lateral spine, active miracidium
Fasciola hepatica	140 x 80	Yes, indistinct	Embryo immature, uniform granular structure
Clonorchis sinensis	20 x 4	Yes, distinct	Light yellowish color, prominent shoulders of operculum, embryo fully developed
Opisthorchis felineus	25 x 4	Yes, distinct	Ends narrower and more tapering than Clonorchis, pointed posterior terminal knob
Fasciolopsis buski	135 x 80	Yes, indistinct	Yellowish-brown, embryo immature, clear thick shell, uniform granular structure
Paragonimus westermani	95 x 55	Yes, distinct	Yellowish-brown color, embryo immature

The Lung Fluke, Paragonimus westermani

This lung fluke, fairly common in the Orient, is acquired by eating uncooked infected crabs or crayfish, the intermediate crustacean hosts. After ingestion the flukes are liberated and migrate through the intestinal wall, abdominal cavity, diaphragm, and into the lungs. Once in the lung, they tend to locate near a bronchus, where they develop into the adult worm in 5 or 6 weeks. Gradually a small cystic cavity develops about the worm which ruptures into a bronchus releasing the fluid and many ova. The parasites may lodge in other internal organs or the brain. In man the infection is usually light and the lung cysts predominate. The individual cysts are 0.2 to 1 cm. in diameter, have a whitish fibrotic capsule and inside a brownish gelatinous exudate containing the ova and usually only one worm.

Identification. The laboratory identification is made by demonstrating the ova in sputum. The sputum is apt to be viscid and reddish-brown. Ova also are found in the feces in about 40 per cent of the cases from swallowed sputum or from intestinal lesions. These ova are yellowish-brown in color, operculated but without distinct shoulders,

and contain an immature embryo showing yolk cells and a central germinal area (see Table 14.15).

The Nemathelminthes (Roundworms)

The second group of helminths of medical importance are the Nemathelminthes or roundworms. Only one class causes human infections, the Nematoda. These roundworms are characterized by a symmetrical, smooth, long, non-segmented, slender, cylindrical or filiform body with tapering ends rounded anteriorly and pointed posteriorly. They all possess intestinal tracts and the sexes generally are separate.

There are a vast number of such roundworms and many are free-living. Fourteen of these worms cause medically important infections which can be identified by laboratory methods. For convenience they may be divided into two groups, those primarily intestinal parasites and the filaria, primarily blood and lymph parasites.

THE INTESTINAL ROUNDWORMS

This group comprises seven strictly intestinal parasites and *Trichinella spiralis*, the adult worms of which exist for a short period in the intestinal tract. However, within the same host the larvae migrate to striated muscle and encyst.

Table 14.16 shows the eight intestinal roundworm parasites, the diseases they cause, their hosts, and how man acquires these infections.

Trichinella spiralis

This namatode is important because its larvae encyst in the striated muscles of man, causing the disease trichinosis. The larvae are ingested in insufficiently cooked pork, the adult male and female worms becoming mature within the intestines in 2 or 3 days. The female protrudes its head into the mucosa of the small intestine and gives birth to larvae which enter the blood and lymph vessels and are carried to all parts of the body. The adults are practically microscopic in length (male 1.5 mm.; female 4 mm.). They remain in the intestine only 3 or 4 weeks. If the disease is suspected during this period they may be found in the stools following a purge. After repeated washing and centrifugations the worms may be found in the sediment of such purged stools. The males are identified by two conical papillae protruding from the posterior end. The female may be identified by the presence of eggs in the posterior part and larvae in the anterior part of the uterus, which terminates in the vulva at about one-fifth the distance from

TABLE 14.16

Eight Intestinal Roundworm Infections

Genus and Species	Disease	Definitive Host	Intermediate Host	Infection Acquired
Trichinella spiralis	Trichinosis	Man, hogs, rats	Man, hogs, rats	Ingestion of poorly cooked infected pork
Trichuris trichiura	Trichuriasis or whipworm infection	Man	None	Ingestion of ova
Strongyloides stercoralis	Strongyloidiasis	Man	None	Larvae penetrate skin
Ancylostoma duodenale	Hookworm infection	Man	None	Larvae penetrate skin
Ancylostoma braziliense	Creeping eruption	Dog, cat*	None	Larvae penetrate skin
Necator americanus	Hookworm infection	Man	None	Larvae penetrate skin
Enterobius vermicularis	Enterobiasis or pinworm infection	Man	None	Ingestion of ova
Ascaris lumbricoides	Ascariasis or roundworm infection	Man	None	Ingestion of ova

* Man is an accidental host.

the anterior end of the worm. The larvae can be found after the second week of *symptoms* in biopsy specimens encysted in the deltoid or gastrocnemius muscle near the tendinous end in about 50 per cent of clinical cases. The biopsy specimen should be about 1 cm. square by 0.5 cm. thick. It should be examined by cutting it into small pieces and pressing these out in thin layers between glass slides. The larvae can be seen with a low power of the microscope coiled up in the muscle fibers.

The digestion method is simple, accurate, and rapid. Trichinella larvae are found four times more often with it than with a modified serial section method and three times more often than by the press method. The basis of the test is the separation of dead muscle from the living trichina by digestion of the muscle with artificial gastric juice. The living larvae are not affected by the gastric juice (see Fig. 14.9).

Other aids in the diagnosis of trichinosis are by means of the precipitin and complement fixation serological tests and the skin tests (see Chapter 9).

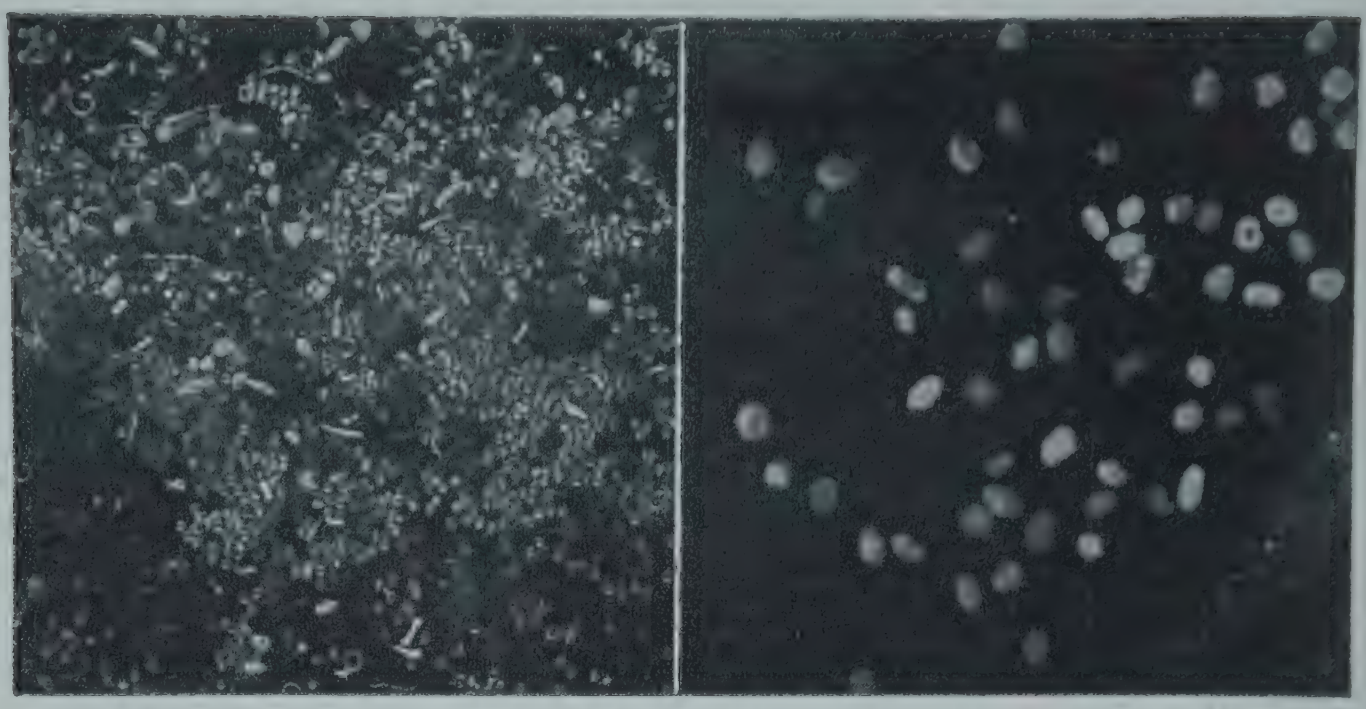


FIG. 14.9. 1. Isolated living *Trichinella* larvae (× 14), (Reduced $\frac{2}{3}$). 2. Isolated *Trichinella* cysts (× 14), (Reduced $\frac{2}{3}$). From Am. J. Clin. Path. 9:212, 1939.

Interpretation. Trichinosis is a very common disease in the United States. Surveys of human autopsies indicate that about 19 per cent of the population is infected. Many of these infections are so light that they probably produce no clinical symptoms, but many clinical cases undoubtedly remain undiagnosed. The number of diagnosed cases has increased markedly during the past few years. A positive biopsy is the only absolute method of diagnosis but is positive in only 50 per cent of clinical cases. Most cases show a high eosinophilia but a small proportion do not. The skin test is very specific and is positive in almost all cases after the second week of the disease. In a few very severe cases it is negative. The serologic tests, precipitin, and complement fixation tests are also very specific but may not become positive until the fifth week of the disease. It must be remembered that persons with past infections will give positive skin and serologic tests and positive biopsies long after the acute phase of the clinical disease, so that the present history and clinical symptoms must be carefully considered in interpreting these tests.

Trichuris trichiura (*Whip Worm*)

The adult is 30 to 50 mm. in length, with the anterior three-fifths of the body much more slender than the posterior portion. It usually inhabits the cecum, and sometimes the appendix, lower ileum or colon. The anterior portion of the worm is partly threaded into the mucosa of the intestine with the head protruding into the lumen. Eggs in stools are in the one-cell stage, the larvae becoming fully developed within the

egg in two weeks or more. When mature eggs are swallowed, by man, the larvae escape in the small intestine and pass directly to the cecum where they attach themselves to the intestinal wall and develop into adult worms.

The egg (Fig. 14.10 [fig. 7]) is rather slender, oval in shape, with a round plug at each end, making its appearance very characteristic. The average size is 50 by 25 micra. The color is brown and the shell smooth, thick, with double contour. The embryo within the shell may fill the entire space or may be separated from the shell at the ends. The enbryo is usually in the one-cell stage (see Table 14.18).

Strongyloides stercoralis

The life cycle of this parasite may be similar to hookworm but the rhabditiform larvae may develop into free-living adult males and females which in turn give rise to eggs and rhabditiform larvae which develop into infective filariform larvae (see Table 14.16). Sometimes filariform larvae develop in the large intestine of man or about the anus, and penetrate the wall of the intestine or skin, producing autoinfection.

The adult parasitic female, 2 mm. in length, inhabits the wall of the small intestine. Parasitic males are not known to occur. The eggs are practically indistinguishable from those of hookworms. They are delivered into the lumen of the intestine with the larva partly developed. Although eggs are sometimes found in stools, they usually hatch in the intestine and most frequently the rhabditiform larvae are found in stools.

In examining stool specimens an extra smear, thicker than that for eggs, should be made routinely for Strongyloides larvae, since the larvae may not be numerous. A rapid examination under low power will easily detect live larvae because of their large size and active movement. The first stage (rhabditiform) larva measures 250 by 350 micra in length by 16 micra in diameter (Fig. 14.10 [fig. 3]). The anterior end is rounded, the posterior end pointed. The buccal cavity is short—less than half the diameter of the body. The genital anlage, located just posterior to middle of the body, is a large, oval cell. Movement is vigorous and snake-like, but not very rapid. In old stool specimens, or if there is constipation, the larva may have passed through one or two moults. After two moults it may become a free-living male or female, which very rarely are found in laboratory stool specimens. Or it may become an infective (filariform) larva, more often found in laboratory stool specimens. The filariform larva measures 450 to 650 micra in length by 25 micra in diameter, the buccal cavity having disappeared.

FIG. 14.10



The esophagus is one-half the length of the body, while the genital anlage is not prominent as it is in the rhabditiform larva. The tail is notched at the tip and movement is more active than that of the rhabditiform larva.

Strongyloides larvae, both rhabditiform and filariform, may have to be differentiated from the corresponding stages of hookworm larvae. The chief differential points are shown in Table 14.17, and the buccal capsules of the rhabditiform larvae in Figure 14.11 [figs. 1 and 2].

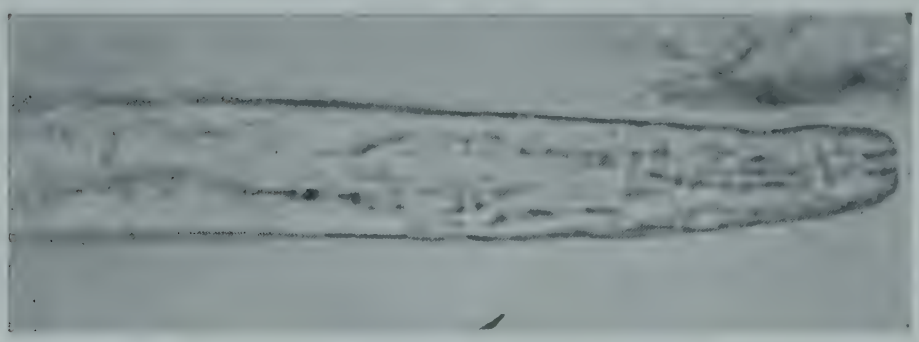
Ancylostoma duodenale, *Necator americanus* (*Hookworm Infection*) and *Ancylostoma braziliense* (*Creeping Eruption*)

Hookworm infection is an old disease of man in tropical and subtropical climates. The infection is acquired when the bare skin comes into contact with infected soil. The fine filariform larvae (see Table 14.17) penetrate the unbroken skin, are carried by the blood to the lungs where the larvae ascend the bronchi and trachea, are swallowed, and in the small intestines develop into the adult worms. A few worms produce no symptoms, but heavy infections produce symptoms of a chronic anemia with weakness.

The adult worms are small—5 to 13 mm.—curves worms which attach themselves to the intestinal mucosa by means of a strong buccal capsule and suck blood. The characteristic ova are about 60 by 40 micra, ovoid, with bluntly rounded ends. The shell is thin, smooth and transparent, while the embryo usually is in the 4- or 8-cell stage (see Table 14.18).

FIG. 14.10. Eggs of intestinal worms. Photomicrographs at magnification of 400.

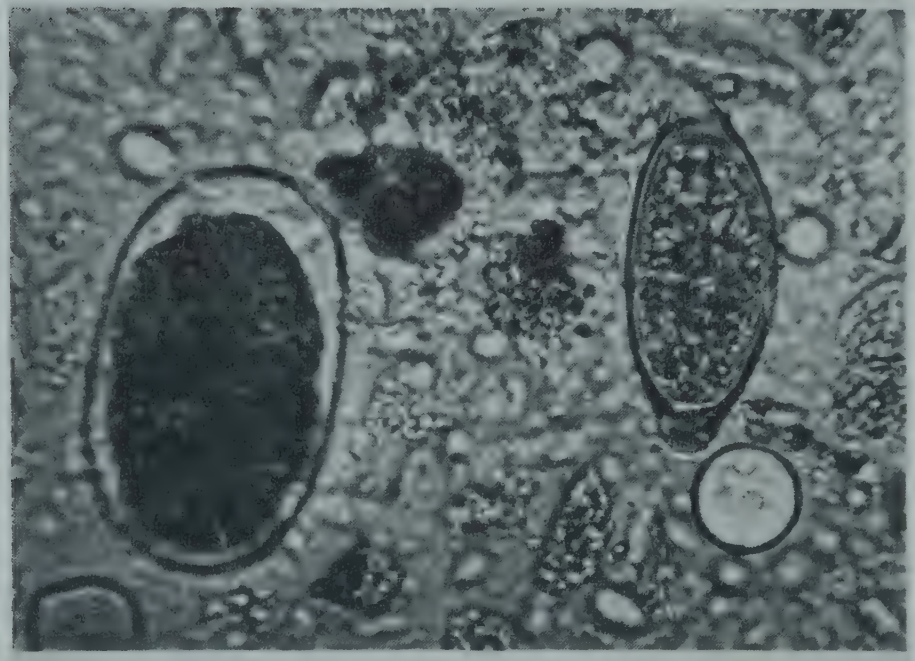
Fig. 1. Egg of hookworm, one-cell stage.
Fig. 2. Egg of hookworm eight-cell stage.
Fig. 3. Rhabditiform larva of *Strongyloides stercoralis*. Note short buccal capsule and large genital anlage.
Fig. 4. Fertilized egg of *Ascaris lumbricoides* with capsule.
Fig. 5. Fertilized egg of *Ascaris lumbricoides* without capsule.
Fig. 6. Unfertilized egg of *Ascaris lumbricoides* with capsule.
Fig. 7. Egg of *Trichuris trichiura*.
Fig. 8. Egg of *Enterobius vermicularis*.
Fig. 9. Egg of *Taenia* with outer shell.
Fig. 10. Egg of *Taenia* without outer shell.
Fig. 11. Egg of *Hymenolepis nana*.
Fig. 12. Egg of *Hymenolepis diminuta*.
Fig. 13. Egg of *Diphyllobothrium latum*.
Fig. 14. Egg of *Schistosoma haematobium*.
Fig. 15. Egg of *Schistosoma mansoni*.
Fig. 16. Egg of *Schistosoma japonicum*.
Fig. 17. Egg of *Fasciolopsis buski*.
Fig. 18. Eggs of *Clonorchis sinensis*.



1.



2.



3.

FIG. 14.11. 1. Anterior end of larva of *Strongyloides stercoralis* showing short buccal capsule. 2. Anterior end of larva of *Necator americanus* showing long buccal capsule; its length is equal to diameter of head of larva. 3. Size relationship between ova of parasitic worms (Necator and Trichuris) and protozoan cyst (*E. coli*), × 750.

TABLE 14.17

Differential Characteristics of Two Common Roundworm Larvae

	Strongyloides	Hookworm
Rhabditiform larva (first stage):		
Length of buccal cavity	Less than $\frac{1}{2}$ diameter of larva	About same as diameter of larva
Genital anlage	Large	Small
Filariform larva (infective):		
Length of esophagus	$\frac{1}{2}$ length of larva	$\frac{1}{4}$ length of larva
Tip of tail	Notched	Pointed

TABLE 14.18

Differential Characteristics of Roundworm Ova Found in Feces

Genus and Species	Size	Shape	Shell	Embryonal Contents	Special Characteristics
	μ				
Trichinella spiralis	—	—	—	—	No ova produced
Trichuris trichiura	53 x 23	Barrel-shaped	Smooth, thick, transparent, bile stained	Unsegmented	Semiopaque polar plugs
Strongyloides stercoralis	54 x 32	Ovoid, blunt rounded ends	Smooth, thin, transparent	Ova rarely passed in feces	Rarely seen in feces
Ancylostoma duodenale and *Mecator americanus*	60 x 40	Ovoid, blunt rounded ends	Smooth, thin, transparent	Incomplete, usually 4–16 cell divisions	Unfertile eggs vastly different from fertilized
Enterobius vermicularis	55 x 26	Asymmetrical	Smooth, thin, transparent	Complete embryo	One side convex, other side nearly flat
Ascaris lumbricoides	60 x 45	Ovoid	Rough, thick, transparent, bile stained	Unsegmented or unfertile	Brown outer rough covering characteristic

The ova hatch within 24 hours in moist warm soil into rhabditiform larvae which grow rapidly, moult twice, then become the long, slender, active filariform larvae infective for man.

Ancylostoma braziliense is the ordinary hookworm infection of dogs and cats. The larvae from their feces, upon entering human skin, burrow as much as an inch intra-cutaneously daily, causing the so-called creeping eruption. Exceedingly rarely to these larvae penetrate to internal organs or the adults develop in the intestines of man.

Enterobius vermicularis (*Pinworm, Seatworm*)

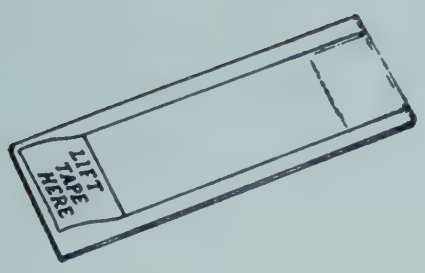
The adult male, 2 to 5 mm. in length, is rarely found. The adult female is 8 to 13 mm. in length. Both adults inhabit the large intestine, and occasionally are found in the appendix. The females migrate to the rectum and pass through the anus to the perianal region, where the eggs are deposited onto the anal folds of the skin. Infection of man takes place by swallowing eggs either on fingers contaminated by scratching skin, by close contact with an infected person, or by swallowing contaminated food or water (see Table 14.16). The larva escapes from the egg in the small intestine, passes into the large intestine and develops into the adult parasites.

The egg (Fig. 14.10 [fig. 8]) is long and oval, with one flat side and relatively slender ends. The average size is 50 by 20 micra, with a smooth, somewhat thicker shell than that of hookworm, and also with a double contour (see Table 14.18). The embryo is usually separated from the shell by a clear space. The larvae may be partly or fully developed. Eggs are not often found inside the stool but may be found by scraping folds of skin radially outward from the anus with an applicator made from a small sheet of cellophane or Scotch tape held over the end of a glass slide. Note simple technique as illustrated in Figure 14.12.

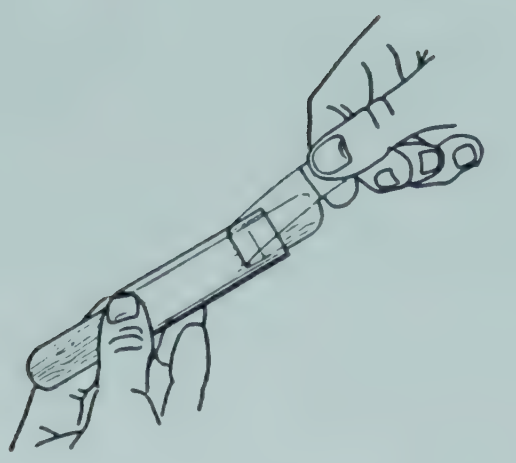
Ascaris lumbricoides (*Large Roundworm*)

The adult worms, 15 to 35 cm. in length, inhabit the small intestine. Eggs, both fertile and unfertile, are found in feces; the fertile eggs contain the embryo in the one-celled stage. Larvae become fully developed within the eggs after three weeks or more in most soil; previous to this time they are not infectious. When eggs containing fully developed larvae are swallowed by man, the larvae are liberated in the small intestine, penetrate the intestinal wall, and are carried by the venous circulation through the liver to the lungs. They escape into alveoli and migrate to the intestine by way of the bronchi, esophagus, and stomach.

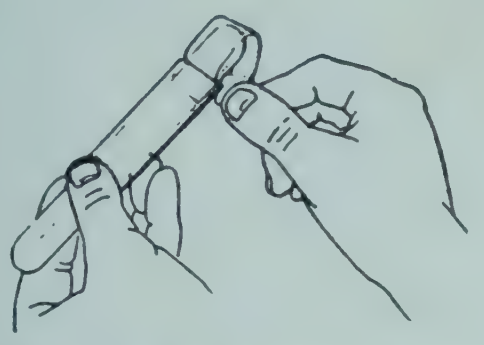
The *fertilized egg* (Fig. 14.10 [fig. 4]) is broadly oval or nearly round and has an average size of 60 by 43 micra. It is covered by an albuminous capsule, irregular in contour and colored brown by the bile pigments in the stool (see Table 14.18). Within this capsule is the thick, smooth, double-contoured shell. The embryo is round or oval, usually in the one-cell stage, and is separated from the ends of the shell by a clear space. In old specimens two- or four-cell embryos may be found. The *unfertilized egg* (Fig. 14.10 [fig. 6]) differs so greatly from the fertilized egg that it may not be recognized as an egg at all. It is long-oval, often



a. Celiulose-tape slide preparation



b. Hold slide against tongue depressor one inch from end and lift long portion of tape from slide



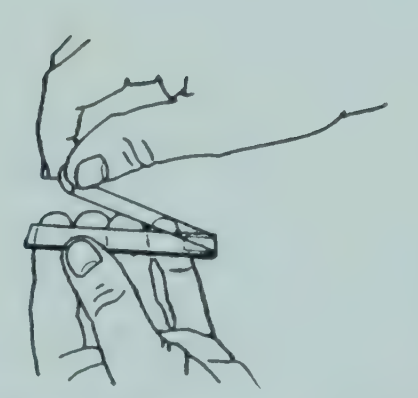
c. Loop tape over end of depressor to expose gummed surface



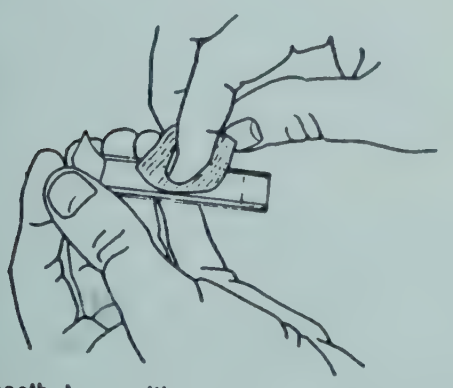
d. Hold tape and slide against tongue depressor



e. Press gummed surfaces against several areas of perianal region



f. Replace tape on slide



g. Smooth tape with cotton or gauze

Note: Specimens are best obtained a few hours after the person has retired, perhaps at 10 or 11 P.M., or the first thing in the morning before a bowel movement or bath.

FIG. 14.12. Use of Cellulose tape slide preparation for diagnosis of pin worm infections. (Adapted from Brooke, Donaldson and Mitchell, 1949.)

with one flattened side or flattened ends. The average size is 75 by 45 micra. The albuminous capsule is more coarsely lobulated than that of the fertilized egg. The shell is thinner and the egg is completely filled with vacuoles in an unorganized stroma. Sometimes either the fertilized or the unfertilized egg may have lost its albuminous capsule, in which case it is colorless and smooth, with the thick double contour of the shell easily visible (Fig. 14.10 [fig. 5]).

THE FILARIA WORMS (FILARIASIS)

There are six filarial worms of medical significance. These slender threadlike roundworms inhabit muscle, connective tissue, serous cavities, the blood, and lymphatic systems of man. They are characterized by a simple oval mouth with little or no buccal cavity and a simple intestinal tract. The females, several times the length of the males, produce embryonated ova that hatch in utero or shortly thereafter. Table 14.19 shows these six parasites, the diseases they cause, endemic area, and how acquired.

The intermediate host, mosquito or biting fly, which is required for development of the life cycle of these parasites, acquires the microfilaria by biting an infected individual. Within the intermediate host, mosquito or fly, the microfilariae undergo metamorphosis into mature larvae but no multiplication occurs. The larvae are transferred by the bite

TABLE 14.19

The Filaria Parasites of Man (Filariasis)

Genus and Species	Disease	Definitive Host	Intermediate Host	Infection Acquired	Endemic Regions
Wuchereria bancrofti	Bancroft's filariasis	Man	Mosquito	Bite of infected mosquito	Tropics and subtropics
Wuchereria malayi	Malayan filariasis	Man	Mosquito	Bite of infected mosquito	East Indies Southeastern Asia
Loa loa	Loaiasis	Man, baboon	Flies	Bite of infected fly	Central and West Africa
Onchocerca volvulus	Onchocerciasis	Man	Flies	Bite of infected fly	Central and West Africa, Central America
Acanthocheilonema perstans	Acanthocheilonemiasis	Man, gorilla, chimpanzee	Midges*	Bite of infected midge	Tropical Africa and So. America
Mansonella ozzardi	Ozzard's filariasis	Man	Midges	Bite of infected midge	West Indies, Central and So. America

* Midges are a group of small two-winged insects resembling gnats.

of the infected mosquito or fly through its proboscis onto the skin of man. The larvae take many months to a few years to mature into adult worms that have quite specific habitats:

Wuchereria bancrofti and *W. malayi*—Lymphatics.
Onchocerca volvulus and *Loa loa*—Subcutaneous tissue.
Acanthocheilonema perstans and *Mansonella ozzardi*—Subserous tissues, abdomen, or thorax.

The term filariasis usually is limited to the infections with *Wuchereria bancrofti* and *W. malayi*, characterized in the early stages by lymphangitis, lymphadenitis, or epididymitis, and later by elephantiasis of the limbs, scrotum, breast, etc., by lymphatic varices of the inguinal region or abdomen, or by chyluria. The subcutaneous nodules containing Onchocerca, if situated on the head, may be associated with blindness. *Loa loa* is possibly associated with transient local areas of subcutaneous edema (Calibar swellings), but otherwise gives rise to no symptoms except consciousness of its presence when passing through the conjunctiva or very thin subcutaneous tissues. *Acanthocheilonema perstans* also may cause temporary localized swellings. *Mansonella ozzardi* is not known to produce any symptoms.

The filaria worms are viviparous; that is, larvae instead of eggs are discharged from the uterus of the female worm. They escape into the vessels or tissues inhabited by the adult worms and enter the blood with greater or less ease, depending on their original location. The larvae of different species vary from 200 to 300 micra in length by 5 to 10 micra in diameter. They have a blunt anterior end and a more or less pointed posterior end, and contain embryonic anlagen of adult organs located at specific points and differing in location in the various species so that identification can be based partly upon their location. The larvae of *Wuchereria bancrofti* (Plate 14.III [Fig. 9]) and *W. malayi* are usually found in the peripheral blood only at night, those of *Loa loa* only during the day. The larvae of the other species have no periodicity. For practical purposes, Table 14.20 is sufficient for identification.

Diagnosis

The larvae (microfilariae) of all except one of the filarial worms are found in the peripheral blood. If numerous enough they can be found in fresh slide coverslip preparations, being easily detected with the low-power objective by their large size and by the agitation of the blood cells. Also, they are easily found in thin blood films stained with Wright's

TABLE 14.20

Distinguishing Characteristics of Microfilariae

Genus and Species	Habitat	Occurrence in Peripheral Blood	Length	Sheath	Posterior End	Special Features in Blood Films
			μ			
Wuchereria bancrofti	Blood	Nocturnal usually	300	Present and may stain	Pointed, no terminal nuclei	Graceful curves
Wuchereria malayi	Blood	Nocturnal	200	Present and may stain	Pointed, two terminal nuclei	Graceful curves
Loa loa	Blood	Diural	300	Present, not stain	Blunt irregular centrl nuclei	Angular curves
Onchocerca volvulus	Skin subcutaneous nodules	Very rare	300	None	Pointed, no terminal nuclei	Not found in blood smears
Acanthocheilonema perstans	Blood	No periodicity	200	None	Blunt terminal nuclei	Blunt posterior, terminal nuclei
Mansonella ozzardi	Blood	No periodicity	200	None	Pointed, no terminal nuclei	Pointed posterior, no nuclei

stain and in dehemoglobinized thick-film preparations. When very few in number they may be found by the following (Knott's) concentration method: To 1 ml. of full strength formalin in a 15 ml. centrifuge tube add 10 ml. of venous blood. Mix until complete hemolysis occurs. Centrifuge and examine the sediment for microfilariae.

Interpretation

The allergic symptoms of infection with *W. bancrofti* or *W. malayi* may begin two months or more after acquiring the infection. Later, the adult worms may be found in enlarged lymph nodes with characteristic histopathological changes present. If the patient leaves the endemic area soon after the early symptoms appear, he will usually overcome the infection without developing permanent deformity and without showing microfilariae in the blood. Some natives of endemic areas, however, show microfilariae in the blood without having elephantiasis or other evidence of lymphatic obstruction, while many of the older infections having lymphatic obstruction show no microfilariae in the blood.

The microfilariae of *Onchocerca volvulus* may be found by aspirating material from nodules containing the worms or by snipping bits of superficial epidermis from the surrounding skin. They are often found at a considerable distance from the nodules.

A skin test using an antigen prepared from the dog heart worm, *Dirofilaria immitis*, gives positive reactions in the large majority of cases of infection with any species of the filarial worms of man. This is a group reaction. A small proportion of apparently false positive reactions occur, some of which may be due to other nematode infections.

Arthropoda

There are a vast number of Arthropods of no medical significance. Only two of the five classes are of interest—the Insecta: the six-legged insects, flies, mosquitoes, etc.; and the Arachnida: having four pairs of legs, the ticks, mites, etc. Most of these Arthropods are of medical significance because they are *vectors* of numerous bacterial and parasitic infectious diseases. The mosquito vectors of malaria and yellow fever, for example, are Arthropods of great medical significance but are not true parasites. Only a few insects and Arachnida are true human

TABLE 14.21

Arthropod Parasites (Infestations)

Genus and Species	Disease	How Acquired	Habitat	Special Features
Trombidium irritans	Dermatitis, chiggers	Bushes and grass	Mostly lower extremities	Removal of chiggers does not help dermatitis
Dermanysus gallinae	Dermatitis	From chickens	Hands, forearms	Scabies-like dermatitis of hands
Sarcoptes scabiei	Scabies	From man	Skin, hands, body	Characteristic burrows, webs of fingers
Pediculus humanus capitus	Pediculosis capitus	From man	Hair of head	The head lice of man
Pediculus humanus corporis	Pediculosis corporis	From man	Under clothing, skin	The body lice of man
Phthirus pubis	Pediculosis pubis	Commonly during sexual intercourse	Pubic hair	The so-call "crab" lice of man
Dermacentor andersoni	Tick paralysis	Bite of tick	Body, skin	Acute intoxication from bite on head or neck of children
Tunga penetrans	Jigger boil	Tropical areas	Hands or feet	Pregnant females bore into skin and develop
Dermatobia hominis	Cutaneous myiasis	Pregnant female bores into skin	Exposed skin surfaces	The so-called botfly boils
Musca domestica (and numerous other species)	Intestinal myiasis	Ingestion of larvae	House fly	Larvae can cause cramps and diarrhea
Cochliomyia americana	Screwworm disease	Deposition of fly eggs in infected wounds	Infected wounds	Prevalent in Central and So. America

parasites, and these generally inhabit the external surface of the body. Such parasitisms are spoken of as infestations.

There are eleven significant Arthropod infestations. Table 14.21 shows their habitat, disease produced, distribution, and how acquired. These are mostly annoying conditions with skin irritation caused by the biting or burrowing of these parasites into the skin. They are generally associated with filth, poor personal hygiene, and general unhygienic living conditions.

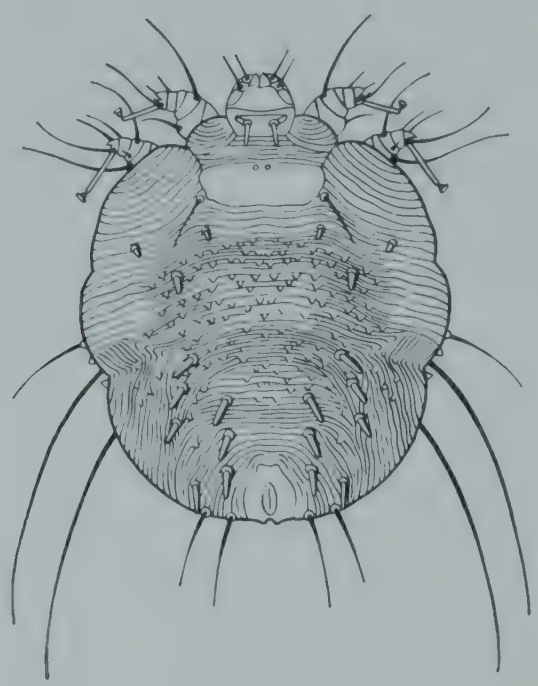


FIG. 14.13 Female scabies mite

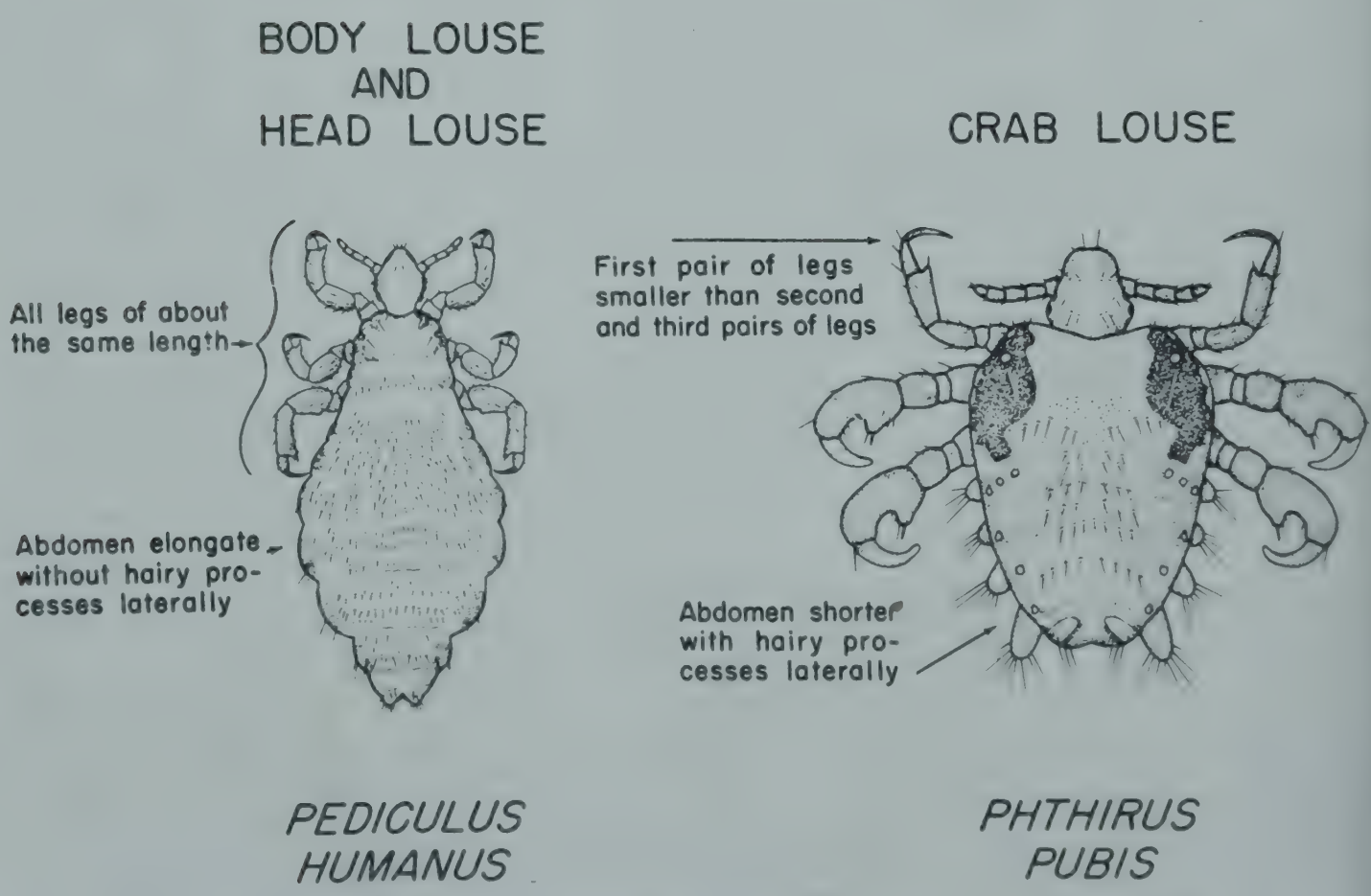


FIG. 14.14. Body louse, head louse and pubic "crab" louse

THE IDENTIFICATION OF ECTOPARASITES—THE ARTHROPOD INFESTATIONS

The examination of these parasites frequently requires the use of a hand lens or low-powered dissecting type microscope. Although the identification of Arthropoda parasites requires extensive entomological training, the clinical laboratory should be able to identify the more important members (see Figs. 14.13 and 14.14).

INTERPRETATION

The identification of a specific parasite establishes a definite etiologic agent causing the infestation and disease. With the effective treatment now available it is most imperative to identify these ectoparasite infestations.

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CHAPTER 15

THE ASSAY OF VITAMINS

By Emmerich von Haam

GENERAL CONSIDERATIONS

Until not so long ago the diagnosis of vitamin deficiency disease had to be made only by the evaluation of the dietary history, the presenting symptoms and signs, and the results of therapeutic trials. In recent years certain laboratory methods have been developed which make it possible to determine the actual vitamin content of tissues and body fluids and thus aid materially in the diagnosis. The available laboratory procedures can be generally classified into three groups: physical and chemical methods of determination and biological assays. The physical methods involve the utilization of ultraviolet absorption spectra, fluorescence spectroscopy, fluorophotometry and polarographic determinations (Loofbourow). Vitamin A, B_6, C, D, E and K have been determined by these methods in various biological materials. The instruments used are generally too complicated and the concentration necessary too high to make physical methods practical for the clinical laboratory. Chemical methods of vitamin determination are being searched for diligently and the recent years have seen a series of microchemical methods come into use which have made it possible to determine the vitamin contents on small samples of plasma obtained by finger puncture. Vitamin A, thiamin, riboflavin, niacine, pyridoxine, choline, folic acid, ascorbic acid and vitamins D_1, E and K can be determined by such chemical methods. For the determination of vitamin A, carotene, riboflavin and vitamin C, accurate micro-methods are now available (Bessey). Table 15.1 lists those vitamins for which practical methods of assay are available.

Vitamin determination by bio-assay is available for practically every vitamin since it was this type of research method which led to their discovery. Two principal methods are used for the determination and quantitative assay of a vitamin in animals. One method is based on the curative effect of the vitamin added in varied doses to the standard diet of animals which have developed recognized deficiency symptoms. The other method tries to prevent the appearance of such deficiency symptoms by adding measured doses of vitamin to the standard diet at the beginning of the experiment. The first method is unquestionably the longer one although the results are more reliable. By this method

TABLE 15.1

Methods for the Assay of Vitamins in Blood and Urine

Vitamin	Chemical Synonym	Functional Synonym	Chem. Det.		Bioassay		Indirect Clinical Evaluation	
			Blood	Urine	Blood	Urine	Type	Value
A	Axerophthol	Antixerophthalmic V.	+	+	—	—	Dark adaptation tests	+
B_1	Thiamine	Antineuritic V.	+	+	+	+	Analysis of diet chart	?
B_2 (G)	Riboflavin		?	+	+	+	Capillary microscopy	?
B_6	Pyridoxine	Antidermatitic V.	—	?	+	+		—
P-P	Nicotinic acid	Pellagra preventive V.	+	+	+	+	Physical examination	?
B_x	Pantothenic acid	Antidermatosis V.	—	—	+	+		—
B_c (M)	Folic acid, pteroyl glutamic acid	Anti-anemic V.	—	—	+	+	Hematologic evaluation	?
B_8	Inositol	Anti-alopecia V.	—	—	+	+		—
B_4	Choline	Anti-paralytic	+	+	+	+		—
B_7 (H)	Biotin	Anti-egg white injury V.	—	—	+	+		—
H	p-Amino-benzoic acid	Chromotrichia F.	+	—	+	+		—
B_{12}	Cyanocobalamin	Animal protein F.	—	—	+	+	Hematologic evaluation	?
C	Ascorbic acid	Antiscorbutic V.	+	+	+	+	Determination of capillary resistance	+
D	Calciferol	Antirachitic V.	—	—	+	+	Determination of calcium phosphorus & phosphatase in blood	+
E	Alpha tocopherol	Antisterility V.	+	+	?	?		—
K	2-methyl-3-phytyl-1,4-naphthoquinone (K_1)	Antihemorrhagic V.	+	+	+	+	Determination of prothrombin time	+
P (C_2)	Flavones	Capillary resistance F.	?	?	?	?		—

+ = practical and reliable; ? = not practical or reliable; — = no method available.

the natural vitamin reserve of the animals does not have to be taken into account since only animals which give organic evidence of vitamin deficiency are used. In the second method a control group of animals is necessary, which are permitted to develop the usual deficiency symptoms. Here the different amounts of vitamin reserve in the various test animals may produce variations in results, requiring a larger group of test animals. Using this principle, assay methods for all known vitamins have been described and properly standardized. In most of the tests, rats are used, and for each vitamin a carefully computed depletion diet has been worked out. These assays, which are used largely by the biological houses for the standardization of their concentrated vitamin products, are too complex for use in a clinical laboratory.

Microbiological assay procedures utilize the rate of growth of certain bacteria as criterion for the vitamin content of the growth medium (Barton-Wright). These microbiological assay methods have been divided into four classes according to the mode of measuring the growth of the microörganisms. They are: the measurement of gas production by yeast fermentation; the measurement of acidity by lactic fermentation; the measurement of turbidity in the medium as related to bacterial growth; and the measurement by weight of the mycelia of molds. The organisms of the *Lactobacillus casei* group have been used most widely as test organisms but other bacteria, yeasts and various molds have also been recommended (Snell). Microbiological assays of vitamins utilize little space and do not require any expensive apparatus. They can be performed by any exact bacteriological laboratory capable of maintaining the stock cultures of the test organisms under strictly standardized conditions. For this reason they should enjoy increasing popularity and should be adaptable for use in larger hospital laboratories.

In the following paragraphs practical laboratory methods available for vitamin determinations in human fluids (blood and urine) will be discussed.

VITAMIN A

General Considerations. The fat-soluble antixerophthalmic vitamin A is present in plants in the form of various provitamins, or carotins. In the animal body part of these carotins are converted into vitamin A, which is stored in the liver, bone marrow, and the subcutaneous fat. Its chemical formula has been established as $C_{20}H_{30}O$, and it is destroyed comparatively easily by fermentative or oxidative processes. Vitamin A is regarded as essential for body growth and the function of

certain specialized tissues such as skin, gums, teeth, hemopoietic system, and the retina. Its deficiency produces hemeralopia, or night blindness, xerophthalmia, eczematous dermatitis, tooth atrophy, and patchy areas of epithelial metaplasia in the bronchi, pancreatic ducts, and vagina. The body suffering from vitamin A deficiency is usually emaciated. Any growth stops during the period of deficiency, and the organism is increasingly susceptible to bacterial infections. It is estimated that the human being needs approximately 5000 units daily, which can be administered either in the form of vitamin or of provitamin, or carotin.

Subclinical forms present numerous difficulties for diagnosis, and laboratory tests have to be employed. A fairly accurate test for storage of vitamin A in the body is to determine the presence and the degree of night blindness. At a cost of less than $5.00, Eddy and Dalldorf constructed an apparatus made from a toy movie projector in which the light source is a small lamp operated by flashlight batteries. An ammeter and 20 ohm variable resistance are connected in series and a simple picture resembling a target is projected on the screen. The light gradually increases in intensity until the patient can recognize the middle circle. The test is then repeated after ten minutes' rest in a dark room. After this period of rest individuals deficient in vitamin A seem little influenced in their ability to see the middle circle in spite of opportunity for regeneration of visual purple. The apparatus must be standardized on normal individuals. Other, more expensive, biophotometers are on the market designed to determine the rate of recovery of acuity as an expression of the rate of regeneration of the visual purple. (Adaptometer of Hecht and Shlaer.)

The chemical determination of vitamin A has been successfully undertaken in food material, pharmaceutical specimens, and body fluids. The standard unit of vitamin A is the effect of 0.6 gamma purified beta carotin. In chemical assays it is sometimes expressed in Lovibund tintometer units, 1 Lovibund unit being approximately equal to 0.2 gamma purest vitamin A or 0.3 gamma standard beta carotin. One Sherman-Munsell growth unit corresponds to 2 Lovibund units. In the urine, sweat, feces, and cerebrospinal fluid, only a minimal trace of vitamin A is found. Considerable progress has been made in the examination of blood and blood plasma concentration.

In the Blood. Chemical determination of vitamin A in the blood employs the principle of the color produced by the vitamin when a saturated solution of antimony trichloride is added (Carr-Price reaction). In the technic of Kaser and Stekol 5 ml. of plasma or serum are required and the vitamin is extracted by shaking it with petroleum

ether for ten minutes. After evaporating the petroleum ether the residue is taken up with chloroform and the color reagent added as quickly as possible. The color is measured in a colorimeter against standardized solutions of copper sulfate or against certain color filters. The maximum of the color development is reached within 5 to 10 sec., after which time the intensity of the blue color diminishes very rapidly. For this reason the measurement of the reaction must be performed quickly and expertly and even small amounts of moisture in the developing extract must be carefully avoided.

A method which guarantees more stability of color, insensitivity to moisture and uses reagents which are easier to handle is based upon the deep purplish color produced by glycerol dichlorohydrin. The procedure has been modified for determination of vitamin A in blood and serum by Sobel and Snow and can be recommended for studies where rather high vitamin levels are expected, as for instance after vitamin therapy.

The normal content of carotin varies between 50 and 300 micrograms per 100 ml. plasma. The vitamin A content varies between 50 and 300 I.U. per 100 ml. plasma. A rapid drop in the plasma level may indicate disturbances in vitamin absorption as occur in celiac disease, sprue, pyloric or intestinal obstruction and liver disease (Popper and Steigmann). A diet deficient in vitamin A usually first produces clinical signs of vitamin deficiency while the plasma level remains normal for many months due to mobilization of stored vitamins from the liver and other tissues.

In the Urine. Normally vitamin A is not excreted in the urine. However in certain pathological conditions, such as kidney and liver disorders, febrile conditions and advanced stages of neoplasms, an excretion of vitamin A takes place, thus creating a depletion of the body of this valuable factor for epithelial growth. It can be determined with the method of Tomaszewski on 50 ml. of urine by extracting the vitamin with petroleum ether and performing the Carr-Price reaction in the extract. For a clinical method a rough classification of the qualitative reaction as 1, 2, or 4 plus is sufficient. For exact data the vitamin content can be determined with the colorimeter as in the blood.

THE VITAMIN B COMPLEX

Thiamine

General Considerations. The water-soluble antineuritic vitamin B (B_1) is present in high concentration in vegetable or plant seeds such as

wheat germ, oats, nuts, and yeast grown on rich media. Chemically it has been identified with thiamine with the chemical formula $C_{12}H_{18}ON_4SCl_2$. It is not very heat-resistant and is sensitive to alkali. One international unit corresponds to 3 gamma vitamin B_1 crystals. Vitamin B_1 is essential for optimum growth of children, for the maintenance of appetite, and for the regulation of certain vasoneurotic reactions. Its deficiency produces beriberi, a disease characterized by cardiac and nervous disturbances leading to generalized edema, paresthesias, paralysis, and inanition. Subclinical forms of vitamin B_1 deficiency will show a confusing array of symptoms which can be recognized only with difficulty. Gastro-intestinal disturbances combined with attacks of polyneuritis and general malaise are frequent. The daily requirement of thiamine has been set by the Food and Nutrition Board of the National Research Council at between 1.2 and 2.3 mg. per day for normal adults in a 2400–4500 calorie diet.

In order to facilitate the diagnosis of vitamin B_1 deficiency numerous attempts have been made to assay the vitamin B_1 level in the urine and other body fluids. Bio-assays have been based on the prevention and cure of polyneuritis in birds and rats, determination of the growth rate, of the weight maintenance, the restoration of appetite, and the appearance of bradycardia and nystagmus. These methods are too complicated and lengthy to be of any clinical value. Methods based on the behavior of plants and microörganisms have been standardized for vitamin B_1 determinations. Chemical tests in use at present are based upon the color reactions with the aromatic diazo reagent, the conversion of the vitamin to thiochrome, and the appearance of bisulphite binding substances (pyruvates) in the blood stream. A comparison between the biological and chemical assays for thiamine shows that usually only 80 to 90 per cent of the thiamine is determined chemically. This is due to thiamine adsorbed on insoluble material produced in the hydrolysis (Brown, Hann and Harrison).

The concentration of vitamin B_1 in the blood is normally around 7 to 8 gamma per 100 ml. For this reason chemical methods have so far not been successfully applied. Instead the indirect evaluation of thiamine levels by determination of pyruvic acid has been preferred. The latter shows an average of 1.0 mg. per cent and becomes elevated in subjects with thiamine deficiency (Jolliffe). For the determination of pyruvic acid in the blood the micromethod of Lu is recommended. For a description of detail we refer the reader to the literature. A rough estimate of the thiamine content in the blood can be obtained by application of the method of Karrer.

Technic:

One half of a ml. of plasma is treated with 0.1 ml. of 1 per cent potassium ferricyanide and 0.3 ml. of 10 per cent potassium hydroxide and after shaking for two minutes is extracted with 12 ml.of isobutyl alcohol. The fluorescence of the oxidized blood sample is compared with the standard extract and from this the vitamin B_1 content calculated. In order to avoid a source of error due to the presence of other fluorescent substances in the plasma, a specimen without potassium ferricyanide is extracted with isobutyl alcohol and examined under ultra-violet light.

In the Urine. The amount of thiamine in the urine is considerably higher than in the blood and purified extracts give usually a strong thiochrome reaction which can be either compared under ultra-violet light with that of standard tubes or measured galvanometrically. The normal excretion of thiamine of persons on a normal adequate diet varies from 100 to 300 gamma in twenty-four hours. Excessive administration of the vitamin leads to increased excretion unless the patient suffers from depletion of the vitamin. Thus it is possible to devise a saturation test and calculate the patient's depletion of thiamine. Of the many methods and modifications we recommend the method of Perlzweig and coworkers as reliable and comparatively simple.

Riboflavin

General Considerations. The water-soluble vitamin B_2, or riboflavin, occurs naturally in many animal and vegetable food stuffs (eggs, milk, animal organs like liver and kidney, lima beans, green vegetables). Chemically it is a derivate of isoalloxazine and has the formula $C_{17}H_{20}N_4O_6$. It is sensitive to alkalis but stable to mineral acids. Its activity used to be expressed in biological units based upon growth response in rats (Sherman-Bourquin unit), but now it is measured in micrograms of the pure substance. Its function is intimately concerned with cell respiration and as a coenzyme it takes part in the cellular utilization of carbohydrates. Deficiency states in man are characterized by vascularization of the cornea, cheilosis and angular stomatitis, glossitis and dermatitis; in animals by curled toe paralysis (chick), weakness and ataxia (dog), dermatitis and keratitis (rat). Clinically its deficiency can be diagnosed by observation of the corneal lesion with the slit lamp.

In the Blood. The quantities of free riboflavin in the blood are extremely small (0.6 to 0.8 gamma per ml. of blood). They can only be measured reliably with the microbiological assay method (Snell and Strong), which is too complex to lend itself to routine laboratory pro-

cedure. For those who are interested in this field the method offers very exact and reliable results and is still simpler than the assay on vitamin deficient animals.

In the Urine. The excretion of riboflavin in the urine has been studied by few chemical and microbiological methods. The daily excretion in the human on an uncontrolled diet varies between 480 and 800 gamma per 24 hours (Strong and coworkers). The method recommended is the fluorometric determination of Najjar. Because of the great sensitivity of the riboflavin to light, all determinations should be performed in a room whose windows are covered with brownish cellophane. Artificial white light should be avoided.

Pyridoxine

General Considerations. The water soluble vitamin B_6, or pyridoxine, occurs naturally in rice, bran, liver, yeast, legumes, egg yolks, cereals, and seeds. Chemically it is a derivative of pyridine and has the empirical formula of $C_8H_{11}O_3N$. It is heat stable and acid fast but is destroyed by ultra-violet radiation. It has growth stimulating action in plants, bacteria, and animals and plays an integral part in the utilization of fatty acids. In man deficiency symptoms are extreme nervousness, irritability, and it is linked with certain central nervous system disorders. In animals deprivation of vitamin B_6 produces acrodynia (a type of dermatitis) in rats, microcytic anemia in the pig and epileptiform attacks in pigs, rats and dogs. The György rat unit is the amount necessary to cure acrodynia in rats. The dose is equivalent to 10 gamma pyridoxine. The clinical diagnosis of deficiency states can not be made and little is known about the actual occurrence and frequency of pyridoxine deficiency. Microbiological and animal assay methods are available for the determination of pyridoxine levels in blood and urine (Landy and Dicken). Chemical methods have been worked out for the determination of pyridoxine in the urine only.

In the Urine. Normally the human urine contains too little pyridoxine to be evaluated quantitatively chemically. However, injection of 100 mg. of the vitamin will produce in the normal human a substantial rise in the excretion one hour after the injection while the individual with vitamin storage depletion will not show this increase. Scudi's method makes it possible to detect this rise by a color reaction between the eluted pyridoxine and 2,6-dichloroquinonechloroimide. For details of this method the reader is referred to the original article by Scudi.

Nicotinic Acid

General Considerations. The water-soluble vitamin P-P, or nicotinic acid (niacin) occurs naturally in liver, pork, beef, milk, egg yolk, spinach, and lettuce. Chemically nicotinic acid is a derivative of pyridine and has the chemical formula $C_6H_5O_2N$. It is heat stable. Physiologically it is the constituent of a coenzyme system necessary for normal cellular life. Its mechanism seems to influence vitally the physiology of epithelial and nervous tissues. It is measured in milligram units. Deficiency in the human is characterized by the development of the disease complex of pellagra with its typical dermatological, intestinal, and nervous manifestations. Deficiency states in the animal are characterized by black tongue (dogs) and dermatitis and diarrhea (pigs). The diagnosis of niacin deficiency rests mainly upon the recognition of the typical clinical picture. The excretion of porphyrins in the urine is not considered a reliable diagnostic sign. Microbiological and animal assays are available for biological material. Chemical methods for the determination of niacin in urine and blood have been worked out. However, they have at present no value for the clinical diagnosis of deficient states. Pellagrins and normal individuals show the same range of blood niacin levels, and the determination of the pellagra prevention factor in the urine is handicapped by the appearance of other substances, especially trigonelline, giving the same color reaction. Those who are interested in niacin assays in spite of the adverse criticism here expressed may be referred to the following methods of assay which are now being used in the literature.

In the Blood. Nicotinic acid can be determined in erythrocytes, plasma and serum by the method of Klein, Perlzweig and Handler. Normal values range between 0.27 and 0.80 mg. per cent.

In the Urine. One of the widely used methods is the one of Vilter, Spies and Mathews, which utilizes the Koenig reaction for the determination of niacin. The normal excretion values are given as 20 to 50 mg. daily. Their method is criticized sharply because of the failure to include conjugated niacins. Attempts have been made to increase the diagnostic value of urinary excretion of nicotinic acid by employing the "load" principle. If an organism is deficient of the vitamin an administration of a large quantity of nicotinic acid (50 to 500 mg.) will not be followed by a corresponding increase in its excretion in the urine. While this test has been effective as a research tool, no standard clinical technique has yet been established (Hundley).

Pantothenic Acid

General Considerations. This water soluble factor of the B Complex occurs as its name indicates universally distributed in plant and animal tissues. Chemically it is a peptide of beta-alanine, has the chemical formula $C_9H_{17}O_5N$ and is stable to light and air but sensitive to heat. It is measured in milligrams. Physiologically it is stimulating to yeast in concentrations as low as 0.0000008 mg. per ml. of culture media. Its deficiency symptoms in the animal consist of dermatitis and adrenal hemorrhage (rat), nutritional achromotrichia (rat), peripheral nerve degeneration (swine). Requirements in man are unknown and no standards have been established for the clinical diagnosis of pantothenic acid deficiency.

In the Blood. Pantothenic acid can be assayed in the blood by the microbiological method of Pelczar and Porter, using the specific growth response of *Proteus morganii* as criterion of vitamin concentration. Its concentration in the normal human is between 0.19 and 0.32 gamma per cc. of blood.

In the Urine. Pantothenic acid is determined according to the method of Pennington and coworkers using the acid production of *Lactobacillus casei* as the assay end point. The normal excretion over a twenty-four hour period varies between 1.74 and 4.30 mg.

Folic Acids

In this group fall a number of substances discovered by various authors under various names which have two biological properties in common. They have a strong growth promoting factor for certain bacteria, notably *Lactobacillus casei*, and also seem to play an important role in the erythrocyte maturation of man. The latter effect has been successfully utilized in the therapy of certain anemias, namely, the macrocytic anemia of sprue, pernicious anemia, myeloblastic anemia of infancy, macrocytic anemia of pregnancy, and so-called nutritional macrocytic anemia (Darby). Chemically many of these active substances have been now identified as derivatives of pteroylglutamic acid (PBA), and Darby suggests the name "pteroyl derivatives" or "pteroylglutamates" as a more suitable designation than the group name, folic acid. They are found either free or in the form of "conjugates" widespread among green-leaf vegetables such as spinach, mushrooms, and are also found in animal livers and kidneys. A deficiency of the factors or of the en-

zyme (conjugase) necessary to produce the active form results in anemia, leucopenia, and weight loss. Although an experimental deficiency of folic acid has not yet been produced in man, Eddy feels that in view of the undoubted activity of the vitamin "it seems probable that it is a definite human requirement." Determination of folic acid concentrations can be undertaken by colorimetric (Glazko and Wolf), fluorometric (Alfrey and coworkers), and polarographic methods (Mader and Frediani). The most sensitive and most exact method of assay is the microbiological assay utilizing *Streptococcus fecalis* or *Lactobacillus casei* as test organism. Growth of the organisms can be measured either turbidimetrically or by titrimetric methods. Jukes and Stokstad recommend the method of Tepley and Elvehjem as the method of choice, particularly for small concentrations of pteroylglutamic acid. In man the excretion of folic acid in the urine is quite low—usually less than 5 micrograms per 24 hours. However, oral administration of PGA raises the urinary excretion in normal patients much higher than in patients with blood dyscrasias (Steinkamp and coworkers). Plasma concentrations of folic acid in man have not yet been reported.

Other Members of the Vitamin B Complex

The following substances fulfill Ansbacher's criteria of members of the vitamin B complex and shall be mentioned briefly. Deficiency symptoms in man are not yet definitely known, although those in animals are fairly well established. Assay methods involve mostly microbiological technics and have at the present time little practical value for the clinical laboratory.

Inositol ($C_6H_{12}O_6$) is a normal cell constituent of practically all plant and animal tissues. Inositol deficiency produces retarded growth and alopecia in rats and mice. It seems to have in some respects a similar action as choline in the prevention of fatty livers, and as vitamin E with regard to the skin and reproduction. Its value in human nutrition still remains unknown. It can be determined by the chemical method of Platt and Glock or the microbiological method using various yeasts or Neurospora as test organisms.

Choline ($C_5H_{15}NO_2$) is present in the phospholipids of practically all cells. Choline deficiency produces fatty liver in rats and dogs with diffuse nodular cirrhosis. In chicks, hens and turkeys it produces perosis, slow growth, and increased mortality. As acetylcholine it influences nerve impulses and lowers the blood pressure. Its place in human nutrition is still unestablished although it has been used widely with

varying success in the treatment of liver cirrhosis. Its excretion in the urine and feces can be measured with the very sensitive microassay method of Horowitz and Beadle. Accurate chemical determination of choline in biological specimens and fluids can be performed by precipitation with Reinecke's reagent and measuring the choline reineckate colorimetrically. This method will determine as little as 15 gamma of choline (Winzler and Meserve).

Biotin ($C_{10}H_{16}O_3N_2S$) is fairly widely distributed in meats and vegetables and is also synthesized by bacterial action in the intestinal tract of man. For this reason deficiency symptoms in man are unknown and no minimal requirement has been established. In animals biotin deficiency is produced by administration of ovoidin rich egg white and produces dermatitis, nervous symptoms, loss of weight, and finally death. Biotin is excreted in the urine 20–50 gamma per day (Oppel), and the total biotin excretion invariably exceeds the intake. Attempts to create biotin deficiency in man have been little successful (Eddy). The best method for assay is the microbiological method using *Lactobacillus arabinosus* or *Streptococcus fecalis* as test organisms (Snell).

Para-amino-benzoic acid (PABA) has the chemical formula $C_7H_7O_2N$ and seems widely distributed among plant and animal tissues. It is best known because of its antagonistic effect to the chemically related sulfonamides, and deficiency in the para-amino-benzoic acid intake causes graying of the fur in the black rat and disturbance in the development of the growing chick. It can be chemically determined by the method of Eckert and by the microbiological assay method of Lewis. Clinically PABA has been used successfully in the treatment of louse-borne typhus and other rickettsial diseases. The normal human requirement of this member of the vitamin B family is unknown.

Cyanocobalamin (vitamin B_{12}) was discovered in 1948 as the long sought "anti-pernicious anemia factor." It is contained in eggs, milk, meat and particularly liver and has the empirical formula of $C_{63}H_{84}N_{14}O_{14}PCo$ (Harris). Normal absorption of the vitamin seems to depend upon the presence of an intrinsic factor excreted by the stomach. Its administration to patients with pernicious anemia is promptly followed by increased erythrocyte regeneration with rise in the number of reticulocytes and the hemoglobin content of the blood together with marked relief of the nervous symptoms, the lingual manifestations and the appetite of the patients (Wolf and Folkers). It also proved effective in other related nutritional anemias such as sprue and nutritional macrocytic anemia. The pathological manifestations of vitamin B_{12} deficiency

are so interrelated with those of folic acid deficiency that usually no clear differentiation can be made (Bethell). Its principal manifestation is an anemia characterized by a megaloblastic type of erythropoiesis. Man's requirement of vitamin B_{12} has not yet been established since it is undoubtedly synthesized by intestinal bacteria when lacking in food. For its determination in the blood and urine the microbiological method of Skeggs and coworkers can be recommended. It utilizes the *Lactobacillus leishmannii 4797* as test organism and has a sensitivity of 0.1 gamma per milliliter of sample. The method is described in detail in the second edition of Vitamin Assay.

VITAMIN C

General Considerations. The water soluble antiscorbutic vitamin C is present in fresh foods and vegetables. Chemically it has been identified with cevitamic or ascorbic acid with the formula $C_6H_8O_6$. Its unique action in the body is due largely to the fact that it is a special kind of reducing agent, influencing the process of oxidation reduction (Harris). In the absence of vitamin C certain cells are no longer able to perform their normal function and, according to Wolbach, cells without vitamin C are unable to produce or maintain the important intercellular substances. It is estimated that a daily intake of 2.5 mg. of ascorbic acid for infants and 7.5 mg. for adults is sufficient to prevent symptoms of deficiency. The principal result of such deficiency is scurvy, a disease characterized by weakness, pains in the extremities, bleeding gums, and subcutaneous hemorrhages.

Subclinical cases of vitamin C deficiency are quite common among the poorer population of large cities. The symptoms are vague and usually limited to disturbances of tooth growth, gum lesions, and slight anemia. An excellent clinical test for vitamin C deficiency is the determination of the average capillary resistance. This can be done with a simple blood pressure apparatus, with which the pressure within the capillaries is increased by constricting the arm veins. Hemorrhages in the skin appearing in excessive amount will indicate capillary fragility. Various attempts at standardization are difficult because of individual as well as anatomical variations of this capillary resistance. Decolorization of intradermally injected sodium 2-6 dichlorophenolindophenol has been advanced as a rapid clinical test for the saturation of tissues with the vitamin (Slobody). Five-hundredths cc. of a N/300 solution of the dye are injected intracutaneously and the time of the disappearance of the blue color noted. A skin test time of more than 14 minutes suggests

a definite degree of tissue depletion; of from 9 to 13 minutes, a mild degree of depletion; and of less than 9 minutes a normal vitamin content of the tissues.

In the Blood. For the determination of vitamin C in body fluids, Tillmans' reaction is the most commonly used. It consists in the decolorization of the pink color with 2-6 dichlorophenolindophenol. A great number of different modifications have been introduced to overcome the presence of other plant and animal materials. It is important that the blood be prevented from clotting by transferring it immediately into a bottle containing lithium oxalate. The plasma is then pipetted off, deproteinized with metaphosphoric acid and is ready for assay. We have found the method of Magnusson and Osterberg reliable and comparatively simple. The photometric measurement of the indophenol xylene reaction as proposed by Robinson and Stotz is capable of widespread application with good correction for various interferences (György and Rubin). From 0.7 to 1.4 mg. per cent may be considered the normal value for ascorbic acid or vitamin C in blood plasma.

Another method recommended for the determination of vitamin C in blood is the microtitration method of Farmer and Abt, using only 0.3 ml. blood and especially applicable to studies in children. A microchemical method using only 0.01 ml. of serum has been introduced by Lowry, Loper and Bessey as a modification of the dinitrophenylhydrazine method of Roe and Kuether. This method is well suited for mass determinations and has proven very effective in our pediatric nutritional survey.

In the Urine. The excretion of vitamin C in the urine depends primarily upon the relative degree of tissue saturation. As the state of tissue saturation is approached the quantity of ascorbic acid increases until at saturation a marked abrupt elevation occurs. The method for the determination of ascorbic acid in the urine is the technic recommended by Harris and Ray. A measured amount of standardized dye is titrated with the urine to discharge its color, the titration being carried out rapidly and in an acid solution. The normal daily output of vitamin acid in the urine of adults varies between 10 and 30 mg.

The colorimetric methods of Roe and Kuether and of Stotz can also be applied for urine. For details see the original literature.

VITAMIN D

General Considerations. The fat soluble vitamin D, or antirachitic vitamin, occurs naturally in egg yolk, butter, cream, and fish liver oils.

The vitamin D complex is composed of at least two active sterols, activated ergosterol or calciferol (vitamin D_2) with the chemical formula $C_{28}H_{44}O$ and activated 7-dihydrocholesterol (vitamin D_3) with the chemical formula $C_{27}H_{44}O$ (Harris). Therapeutic vitamin D_2 is now prepared synthetically by electronic activation of ergosterol. Vitamin D_3 is mainly contained in the fish liver oils. They differ only slightly in their biological activity. The physiological effect of the vitamin D is not fully understood. Apparently it has an influence upon the calcium-phosphorus metabolism, the distribution of phosphatase and perhaps plays a role in cell respiration. Its activity is measured in International Units, which are defined as "the vitamin D activity of 1 mg. of the International standard solution of irradiated ergosterol, which has been found equal to that of 0.025 micrograms of activated 7-dehydrocholesterol." One International Unit is equal to 1 USP unit and approximately equal to 3.3 Steenbock units. Deficiency states in animals and man produce the classical picture of rickets and osteomalacia. Experimental hypervitaminosis produces premature cartilage calcification, marblebone disease, and calcium deposits in organs and blood vessels. For the determination of vitamin D levels in the organism only indirect and biological assay methods are available. The indirect methods include determination of calcium, phosphorus and phosphatase in blood and x-ray examination of the bones. In severe vitamin deficiency the serum inorganic phosphorus is low (less than 4 mg.), the serum calcium may be low (less than 8 mg.) and the serum phosphatase is increased (over 5.0 Bodansky units). In hypervitaminosis the serum phosphorus and serum calcium may be increased

In the Blood. The most satisfactory and most specific method for vitamin D determination is the animal experiment (György and Rubin). Biological assays of sera for vitamin D content can be conducted on rats raised on a rachitogenic diet (Warkany). The procedure is tedious and does not lend itself to a routine laboratory method. The colorimetric reactions include the little specific Liebermann-Burchard reaction and the Rosenheim reaction. Campbell adapted the antimony trichloride reagent for colorimetric determination of vitamin D and improved the method to the extent that as little as 1 gamma of vitamin D_3 per ml. can be detected. The great difficulty is the removal of substances which give similar reactions with $SbCl_3$, such as tachosterol. Presently none of these methods can be used in a routine clinical laboratory.

VITAMIN E

General Considerations. The fat soluble vitamin E, or the antisterility vitamin, occurs in particularly abundant amounts in wheat germ oil and cotton seed oil but is also present in leafy vegetables, meats, and eggs. Chemically it is composed of alpha, beta and gamma tocopherols, the alpha tocopherol ($C_{29}H_{50}O_2$) being the most active one. It is stable to heat, alkalis and acids. It is a strong antioxidant. Its physiological function is still little explored. It has been suggested that it influences the endocrine balance and that it is effective in the nuclear metabolism during the stages of cellular proliferation. Deficiency symptoms in animals include degenerative lesions in the testicles of male rats and of the uterus and the fetal tissues of female rats. Another early sign of vitamin E deficiency is dystrophy of the cross-striated musculature with degenerative lesions of the central nervous system. In chickens vitamin E deficiency leads to fatal encephalomalacia and exudative diathesis. Its evaluation for the nutrition of man is still difficult and experimental. Color reactions with alpha tocopherol have been obtained with alpha bipyridil, concentrated HNO_3 and gold chloride. They form the basis for chemical assay methods in the serum.

In the Blood. The principle of the determination of alpha tocopherol in blood serum is the isolation of the vitamin from all other impurities so that the sensitive iron bi-pyridil color reaction is not interfered with by other substances. The method of Mayer and Sabotka and that of Minot strive to fulfill this aim and both can be recommended for chemical assay of vitamin E. Using hydrogenation to prevent interference by carotenoids and vitamin A, Quaife and Harris introduced a modification of the Emmerie-Engel reaction which is fast and quite precise. Later with Scrimshaw and Lowry, Quaife described a method requiring only 0.06 ml. of blood serum or plasma which consists of measuring and subtracting the light absorption due to the carotenoids from the absorption values obtained by the Emmerie-Engel reaction. The serum or plasma content found in normal individuals averages about 1 mg. per 100 ml. serum.

VITAMIN K

General Considerations. The fat soluble vitamin K, or coagulation vitamin, occurs in nature in the green leaves of plants such as spinach and cabbage, in animal fats and egg yolk. It is also produced by the action of bacteria in the intestinal tract. It is chemically a derivative of

naphthoquinone and at least two natural forms have been isolated: K_1 ($C_{31}H_{46}O_2$) and K_2 ($C_{41}H_{56}O_2$). The synthetic product menadione ($C_{11}H_8O_2$) has the significant naphthoquinone nucleus and shows all the characteristic physiological effects of vitamin K. The principal function of vitamin K appears to be the control of the prothrombin formation. In doing this it plays an important part in the normal mechanism of blood coagulation. Deficiency of vitamin K leads to hemorrhagic conditions which are observed in man and experimental animals alike. In the newborn vitamin K deficiency is responsible for the fatal hemorrhagic disease of the newborn, while in the adult it is an important factor in the production of postoperative hemorrhage observed in patients with jaundice. The diagnosis of vitamin K deficiency is based upon the measurement of the prothrombin content of the blood. This is done by means of the methods of Warner, Brinkhous and Smith and of Quick. For details refer to Chapter 4. The vitamin K content of biological material can be determined by animal assays using the vitamin K deficient chick as test object (Ansbacher). The methods available are too complicated to lend themselves as diagnostic laboratory procedure. Recently a series of color reactions more or less specific for vitamin K have been published, but little progress has been made toward making these reactions applicable for clinical tests (Dam). Kofler recommends a test applicable for urine and plasma based on the color reaction of 2-methyl-1,4-naphthoquinone with cyanoacetic acid ethyl ether. 10 cc. of blood plasma or urine are extracted with ether or petroleum ether and the dried residue of the extract is dissolved in an equal mixture of 95 per cent alcohol and distilled water. The addition of one or two drops of ethylcyanoacetate and 10 per cent ammonia produces a purple color which can be measured with a colorimeter. Scudi and Buhs recommend a colorimetric oxidation reduction method for the determination of the K vitamins in whole blood and plasma which is rapid but which seems to be influenced also by other non-vitamin K substances (Almquist).

VITAMIN P

Vitamin P activity is a postulated effect common to a group of substances known as flavones or flavone derivatives. They occur in various fruits, especially grapes, lemons, oranges and black currants, and are known under various names such as citrin, rutin, eriodictin, quercitrin, and hesperitin. Deficiency symptoms are somewhat related to those of scurvy and include increase in capillary fragility and/or permeability leading to hemorrhagic diathesis (purpura). Quantitative bio-assay

of vitamin P activity is difficult, and impossible in a clinical laboratory. Concerning the various colorimetric methods of determinations, Scarborough and Bacharach state that, "The severe limitations of all such procedures must render almost valueless statements made at the present time about the vitamin P content of biological materials as determined by chemical analysis." To date there exists no specific syndrome of vitamin P deficiency in man and no satisfactory evidence that vitamin P constitutes a dietary requirement (Eddy).

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CHAPTER 16

THE ASSAY OF HORMONES

By Emmerich von Haam

INTRODUCTION

With the increasing importance of hormones in medicine their identification and determination in extracts, body fluids, or tissues are requested more frequently from the clinical laboratory. Many of the methods that have been worked out by various biological institutes are unsuitable for use as diagnostic procedures since they are too costly, complex or unreliable. Simplicity and reliability, however, are absolutely necessary for the successful use of a method in routine laboratory technic.

In order to determine the presence of a hormone, two methods can be used. First, there are indirect methods by which the concentration and activity of the substance to be examined is determined by observing its effect on the human organism. For example, the presence of the female sex hormone can be determined by demonstration of estrus in the female vagina, and the basal metabolic rate gives quite an accurate index of the amount of thyroid hormone produced by the gland. In order to rely upon these indirect methods, it must be assumed that the organs responding to the test react normally, a presumption which may be difficult to prove. The second method of approach, which is by far the more exact one, aims to determine quantitatively the concentration of hormones in the tissues or body fluids by chemical or biological methods. These direct methods have the advantage that they test an isolated hormone outside of the body and thus the usual source of error seen in the indirect method is avoided.

The chemical determination of hormones in body fluids is still in a stage of development too early to be of considerable value. In most instances the chemical composition of the substances to be examined is unknown, and since they are very high molecular organic compounds their analytical determination is extremely difficult and often impossible. The other method of direct determination is to test the substance to be examined on a controlled and prepared group of laboratory animals. This constitutes the principle of the biological assay that has achieved a remarkable degree of standardization. Its accuracy depends upon two factors; first, successful and quantitative isolation of the substance

to be examined by methods of extraction and concentration; and secondly, proper standardization of the test objects. This means the preparation and maintenance of a group of animals that are in equally healthy condition and in the optimal environment for their species with the exception of the substances to be tested. Such conditions can be created for example by methods of experimental surgery such as castration, hypophysectomy or thyroidectomy, or by choosing animals in which this particular hormone has not yet developed or is no longer present. This group includes the use of immature animals for the determination of sex hormones. To small groups of these animals are then administered varying doses of the substance to be examined and the reactions which occur at a given time are then used for the criterion of the potency of the particular substance expressed in units of the animal used. One animal unit represents the smallest amount of hormone which produces in at least 75 per cent of the animals a characteristic and reproducible reaction. It should be emphasized that this reaction may not be identical with the one occurring in the human body. If the substance to be examined has been purified to the extent that it can be obtained in crystalline form, such an animal unit can then be further standardized by using known amounts of the purified substance. As long as this has not been accomplished a certain variation in the quantitative significance of one animal unit will always remain, dependent upon the technic and the strain of animals used by the various investigators. As long as the standardization of an animal unit is impossible, it is therefore absolutely necessary to record in detail the exact procedure used during the bioassay so that a basis for a proper comparison of the results may be created.

Reliable and simple methods for hormone assays are available at the present time only for a small number of hormones while in other instances indirect or clinical methods must be used. Table 16.1 lists all important known hormones and their method of evaluation. In this chapter we shall discuss the determination of the gonadotropic, estrogenic, and luteinizing hormones, the laboratory tests for pregnancy, the 17-ketosteroids, the corticosteroids and the adrenalin.

THE GONADOTROPIC HORMONES

General Considerations. The relationship of the pituitary gland to development of the gonads is well established. The hormone, secreted in the anterior lobe of the pituitary gland, probably from the basophil cells, is composed of at least two fractions: one which stimulates the

TABLE 16.1
Present Status of Hormone Assays

Gland	Hormone	Chem. Assay		Bioassay		Indirect Evaluation	
		Blood	Urine	Blood	Urine	Method	Value
Pituitary							
anterior lobe	Growth factor	—	—	—	—	Glucose tolerance test	?
	Gonadotropic factor	—	—	+	+	Functional test of gonads	?
	Thyrotropic factor	—	—	?	?	Functional test of thyroid	?
	Adrenotropic factor	—	—	?	?		
	Lactogenic factor	—	—	?	?		
posterior lobe	Pitocin	—	—	—	—		
	Pitressin	—	—	—	—		
Thyroid	Thyroxin	—	—	—	—	Protein-bound iodine (PBI)	+
						Radioactive iodine uptake	++
						Basal metabolic rate (BMR)	++
Parathyroid	Parathormone	—	—	—	—	Calcium and phosphorus levels in blood and urine	+
Pancreas	Insulin	—	—	—	—	Glucose tolerance test	+
Adrenal							
cortex	Corticosteroid	—	+	?	+	Robinson water test, Thorn test	+
	17-Ketosteroids	—	+	—	—		
medulla	Adrenalin	+	+	+	+	Blood pressure, fatigue tests	?
	Noradrenalin	+	+	+	+		
Testis	Testosterone	—	—	+	+	Testicular biopsy	+
	17-Ketosteroids	—	+	—	—	Examination of semen	++
Ovary							
follicle	Estrone	?	+	+	+	Vaginal smear	+
c. luteum	Progestin	?	+	?	+	Endometrial biopsy	++
Placenta	Gonadotropins	—	—	+	+	Vaginal smear	?

+ = practical and reliable; ? = not practical or reliable; — = no method available.

follicles in the ovary and the seminiferous tubules in the testis, and another which produces stroma hyperplasia and luteinization of the ovary and stimulation of the interstitial cells in the testis. The secretion of the gonadotropic hormone is inhibited by the action of the male and female sex hormones and by this mechanism a balance between these hormones is assured. In castrated individuals or persons with diseased sex glands an excessive amount of gonadotropic hormone will appear in the blood and urine, a fact which can be taken as reliable diagnostic evidence of primary hypogonadism. During pregnancy a gonadotropic pituitary-like hormone appears in large quantities in blood and urine, which if injected into the immature animal will produce stimulation of the sex glands. However, this hormone, which is the basis for the various tests for pregnancy, is chorionic in origin and definite differences between this substance and the gonad-stimulating hormone from the pituitary have been pointed out, the most important one being that the chorionic hormone of pregnancy is not a true follicle stimulator.

The assay of gonadotropic hormones in the body fluids is of clinical importance in: (1) the diagnosis of pituitary and gonadal disorders; (2) the diagnosis of pregnancy; (3) the diagnosis of certain malignant tumors which produce a similar gonad-stimulating substance. For the evaluation of the gonadotropic pituitary hormone a quantitative determination is desirable since it represents a normal product of endocrine secretion. It appears in the blood and urine of children around the age of puberty and attains its highest concentration just before maturation of the sex glands, afterwards disappearing from the male urine, but appearing at cyclic intervals in the female urine just before ovulation. With cessation of the function of the sex glands the gonadotropic hormone reappears in excess in the blood and urine of men and women for some time, only to disappear again slowly with progressive senile atrophy of the gland. Its quantitative determination is of great value in differentiating primary and secondary gonadal disorders such as amenorrhea and impotency. Since the hormone has not been purified, standardization has not yet been accomplished, and all data referring to animal units represent relative values, depending upon the technic employed. During pregnancy and in malignant tumors the gonadotropic substances rise very rapidly in the plasma and reach such a level of concentration in the urine that normal or even diluted urine can be used for the assay. Since the presence of this predominantly luteinizing hormone in such high concentration never occurs under normal conditions, a qualitative test such as the Aschheim-Zondek or Friedman test is sufficient for diagnostic purposes.

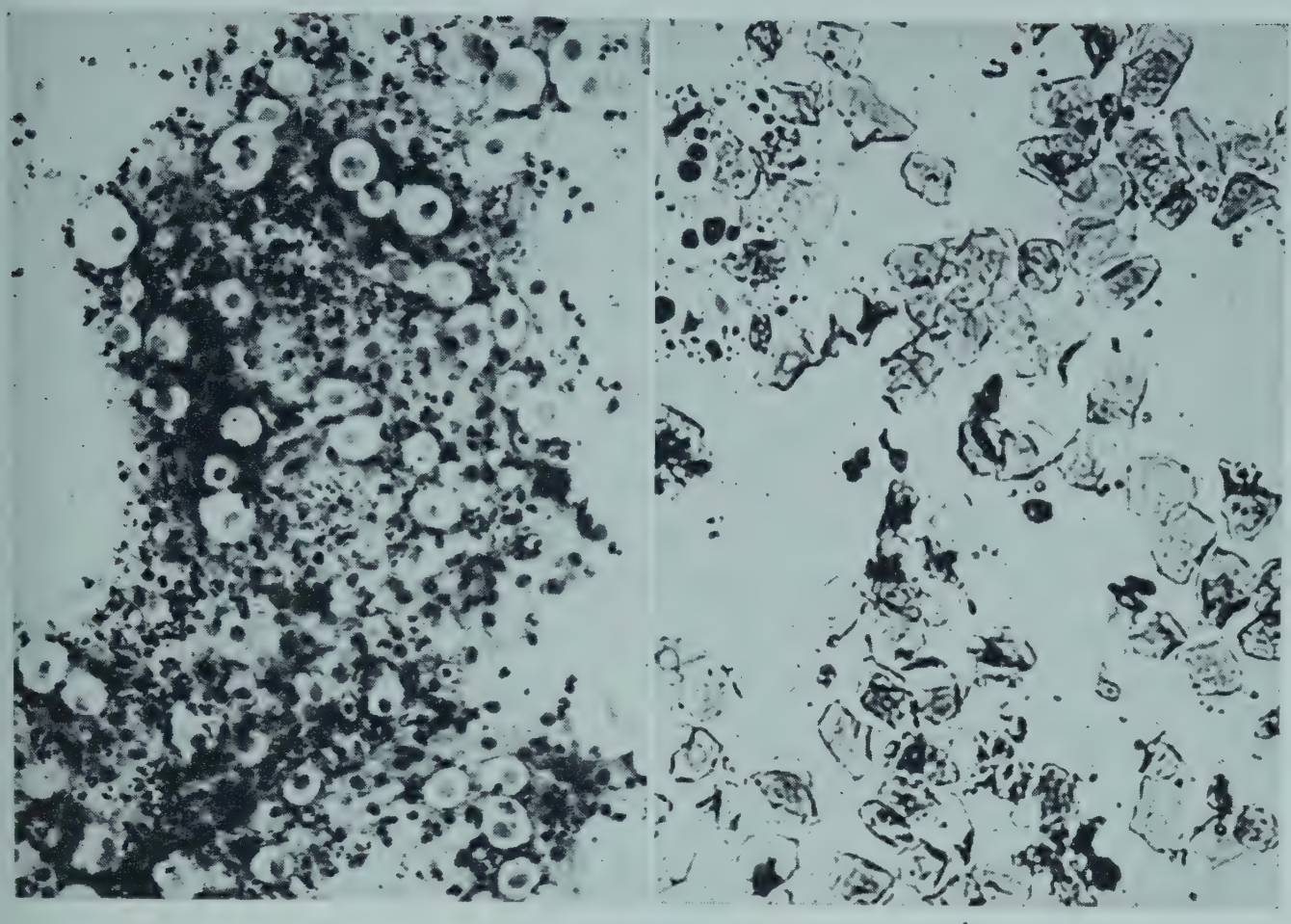
In the Blood. The determination of gonadotropic substances in the blood of nonpregnant women is unreliable because of the small amount present. Normal women, as well as cases of hypopituitarism, show constant negative findings. An increase of gonadotropic hormone in the blood to an extent where it can be demonstrated is, according to Fluhmann, always suggestive of endocrine pathology. This may be primary ovarian weakness or true hyperpituitarism with ovarian hyperfunction. In pregnant women the blood plasma contains a gonadotropic factor which is characterized by its marked luteinizing effect. For the crude assay of gonadotropic hormone in the blood Fluhmann's method is satisfactory. It consists in the injection of varying amounts of serum into immature female mice and histologic examination of the ovaries of the animals five days after the first injection. The ovulation reaction consists in the appearance of a large number of normal ripening follicles; the lutein reaction consists in a marked luteinization of ripe or immature follicles (Fig. 16.1).

In the Urine. The determination of the daily excreted amount of pituitary gonadotropins in the urine has become quite important in the differentiation of primary or secondary hypogonadism. It involves the extraction and purification of the gonadotropic factor from the fresh 24-hour specimen of urine, which may be accomplished either by alcohol or tannic acid precipitation (von Haam). Recently we have used Gorbman's method of filtering urine under pressure through a collodion membrane with good success. The gonadotropins are retained by the membrane, which later is dissolved by alcohol ether. From the residue the active principle can be eluted in a fairly pure and non-toxic condition and assayed on immature or hypophysectomized animals. In over 1000 assays we have found the determination of the increase in uterine weight in the immature female mouse killed four days after the first injection (Levin and Tyndale) an accurate and sensitive mode of assay. An increase in uterine weight over 15 mg. in a mouse not weighing more than 12 gm. can be considered positive (Fig. 16.3). Equally useful but less sensitive is the determination of the estrus by vaginal smear provided the vaginal opening has become patent. The cornified vaginal epithelial cells can be easily distinguished from the basal cells predominant in the vaginal smear of the immature or castrated animal (Fig. 16.2). Other criteria used as end-points for biological assays of pituitary gonadotropins in the human urine are the weight and histological appearance of the ovaries of immature rats or mice and histologically demonstrable changes in the vaginal mucosa of immature cats (Hamburger). The results obtained are expressed in Mouse or Rat Units, and it is manda-



a b c

FIG. 16.1. Microphotograph showing: (a) section through an ovary of an immature mouse; (b) section through an ovary after follicular stimulation; (c) section through an ovary showing the "lutein" reaction.



a b

FIG. 16.2. Microphotograph showing (a) the typical vaginal smear of an immature or castrated animal; (b) of an animal in estrus.

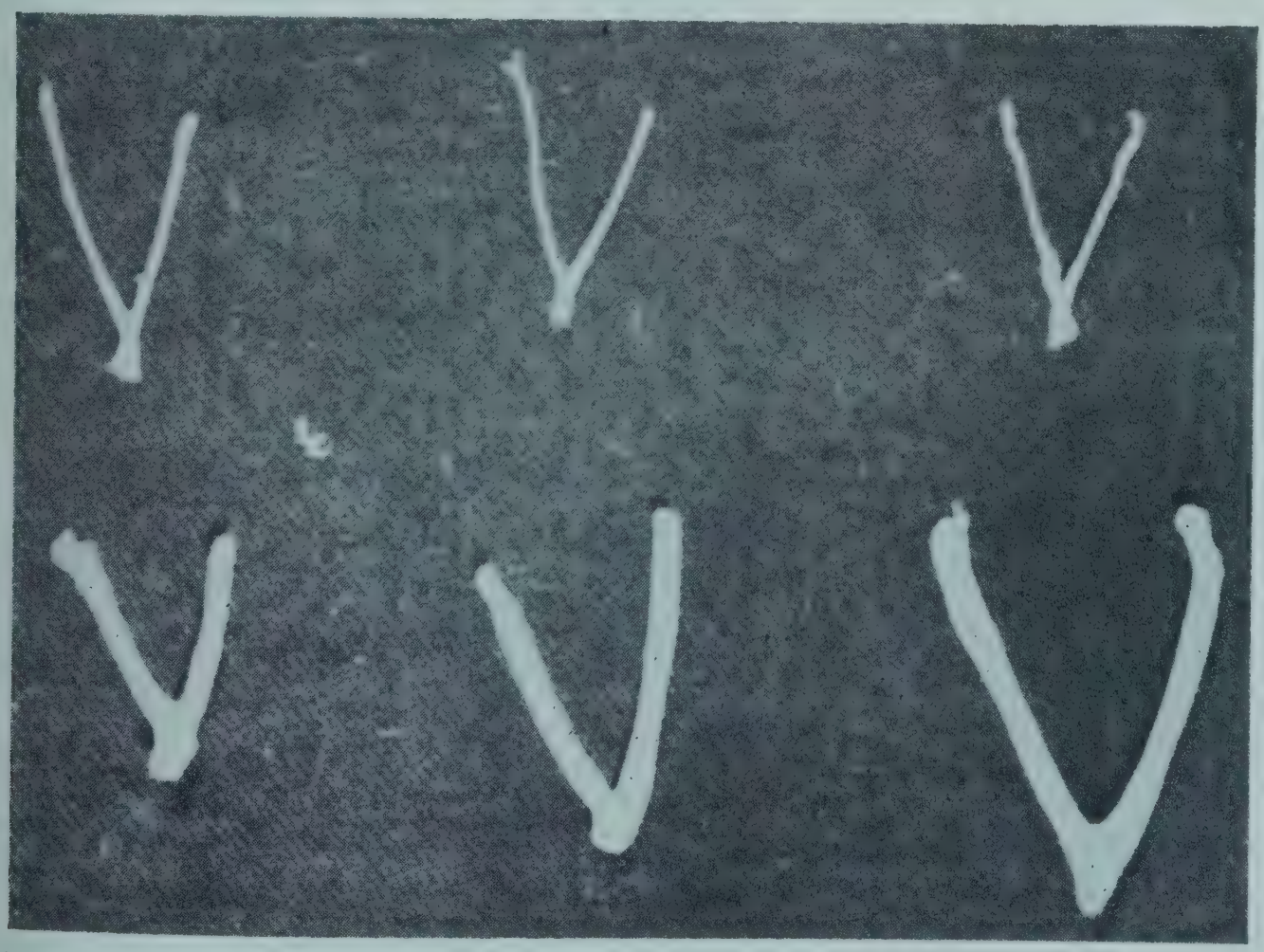


FIG. 16.3. Photograph showing the marked enlargement of infantile mouse uteri after follicular stimulation.

TABLE 16.2

Values of Gonadotropins Obtained in Urinary Assays

Pituitary Gonadotropins 24-hour urine extract dissolved in 24 ml. aqueous solution		Chorionic Gonadotropins in concentrated, normal and diluted specimen per 1000 ml. urine		
Total amount injected	Total excretion in 24 hrs.	Preparation of urine	Total amount injected	Total excretion per 1000 ml.
ml.	*M.U.*		*ml.*	*M.U.*
0.1	240	Conc. 5:1	2	100
0.2	120		1	200
0.3	80		0.5	400
0.4	60	Normal	2	500
0.6	40		1	1,000
0.8	30		0.5	2,000
1.	24	Diluted 1:10	2	5,000
1.2	20		1	10,000
1.6	15	Diluted 1:100	2	50,000
2.4	10		1	100,000
4.8	5		0.2	500,000
			0.1	1,000,000

tory to state which criterion of response was used in the assay. In our experience 1 Rat Unit equalled roughly 10 Mouse Units. For practical purposes we recommend the dilution of the total extract of the 24-hour specimen with 24 ml. H_2O, each cubic centimeter of the final amount corresponding then to the amount of hormone excreted hourly. By injecting amounts ranging from 0.3 to 4.8 ml. of the extract, assays between 80 and 5 M.U. per 24-hour urine specimen can be obtained (Table 16.2). It has been our experience that in normal individuals the level of urinary gonadotropins varies between 0 and 20 M.U. Levels of 40 or over can be definitely regarded as pathological. For a single dose test we recommend an assay at a 60 M.U. level (0.4 ml.). If it is positive it can be concluded that the excretion of pituitary gonadotropins is definitely increased.

Urinary Gonadotropins During Pregnancy and Malignant Tumors. Concentration of urine specimens for the assay of gonadotropic hormones becomes unnecessary when the hormone excretion reaches a concentration of 1 M.U. per ml. or approximately 1500 to 2000 M.U. per twenty-four-hour period. If it goes beyond it, dilution of the sample will be necessary. During pregnancy, hydatidiform mole, chorionepithelioma

and malignant tumors of the testicles the excretion of chorionic gonadotropins is so excessive that special technics have to be used for their determination. In the practice of medicine the determination of chorionic gonadotropins is most frequently used as a test for the diagnosis of pregnancy and in this capacity has become one of the most useful endocrine diagnostic procedures (Riley). In the qualitative Aschheim-Zondek test four infantile mice weighing from 6 to 10 gm. and three to four weeks old are injected with 0.25 ml. fresh and clear filtered acid urine for three consecutive days. On the fifth day the animals are killed and the ovaries examined at autopsy. The appearance of hemorrhagic follicles in any of one of the injected animals is reported as a positive test. More popular than the Aschheim-Zondek test is the Friedman test using the young female rabbit weighing 4 to 5 lbs. or a mature female which has been isolated for at least 3 weeks as assay animal. 10 ml. of freshly collected clear urine are injected in the marginal vein of the ear (Fig. 16.4)

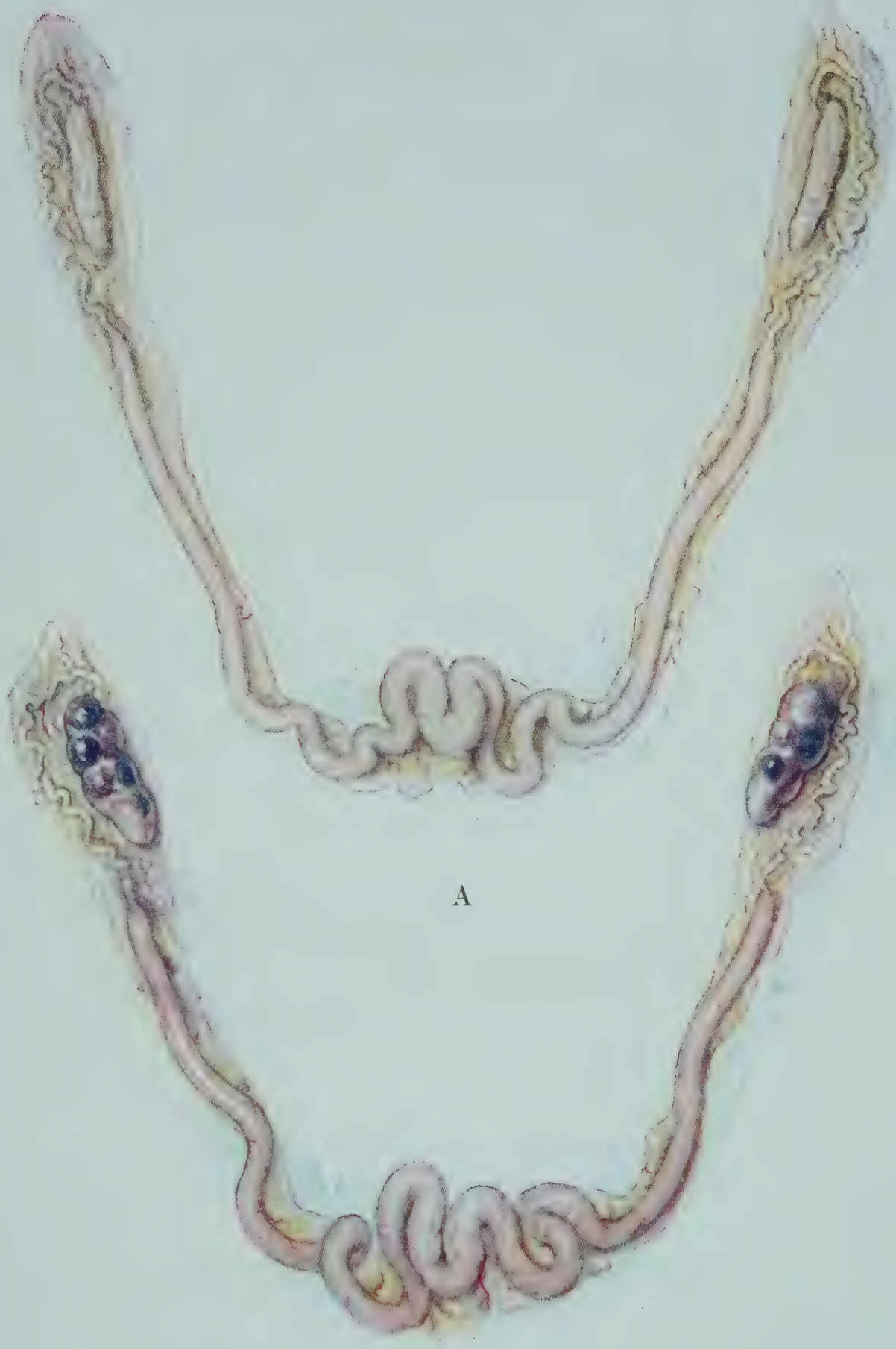


FIG. 16.4. Injecting the marginal ear vein of a rabbit with urine in performing a Friedman test for pregnancy. Note the style of wooden box which restrains the animal in a satisfactory way.

and the animal's ovaries are examined 48 hours later for the appearance of one or more corpora hemorrhagica (Plate 16.I). The test usually becomes positive at a concentration of 10,000 M.U. per 1000 ml. of urine and possesses a high degree of accuracy for the diagnosis of pregnancy from one to three months.

Numerous modification of these tests for pregnancy have been published with all authors striving to make the tests cheaper and faster without losing their reliability. The 24-hour test of Frank and Berman, the 6-hour test of Salmon and coworkers, and the 2-hour test of Kupperman, Greenblatt and Noback are basically modifications of the Aschheim-Zondek principle using the rat as the test animal and hyperemia of the ovaries as the fastest discriminating phenomenon. All authors admit the difficulty in reading the test accurately and for this reason they can not yet be recommended as routine laboratory procedure. Norris recommends the use of whole blood injected subcutaneously into the abdominal wall of rabbits as an equally sensitive and far less toxic method than the Friedman technic. Ten to 12 ml. of whole unclotted blood obtained from the patient's vein 15 days to 3 weeks after the first missed menstruation are used and the test is read in the customary fashion 36 to 48 hours later. Pregnancy tests using the Japanese bitterling and the reaction of the patient against injected colostrum have definitely been abandoned as too unreliable. The newly suggested chemical pregnancy test with 2,4-dinitrophenylhydrazine as color reagent (Richardson) has also failed in the hands of critical observers (Fischer).

A very promising group of animals for the qualitative demonstration of a high concentration of chorionic gonadotropins are members of the family of frogs and toads. Weisman, Snyder and Coates described in 1942 the response of the female South African toad (*Xenopus laevis*) to the injection of concentrated pregnancy urine into the lymph sac by the extrusion of eggs approximately 4 to 18 hours after the injection. Reinhart, Caplan and Shinowara obtained a high degree of accuracy with a cold acetone concentration technic and the male animal of the North American frog, *Rana pipiens*, as a test object. A positive test consisted in the appearance of sperm cells in the frog's urine one or two hours after the injection of the animal. During the reproductive period of this frog (June to August) another common member, *Rana clamitans*, can be used. According to Nieburgs 1 male frog unit is comparable to $\frac{1}{2}$ rat unit, and 1 female frog unit comparable to 5 rat units when testing for chorionic gonadotropins, while 1 male frog unit is comparable to $\frac{1}{12}$ s



A

B

PLATE 16.I. The Friedman test for the diagnosis of pregnancy. A. Bicornuate uterus, tubes and ovaries of a fourteen weeks rabbit thirty hours after injection of urine from a non-pregnant patient. This is a negative test with no changes in the ovaries. B. Showing a positive test consisting of numerous corpora lutea and corpora hemorrhagica in both ovaries. Note the congestion of the uterus as compared with the negative. (From Schneider, P. F.: Surg., Gynec. & Obst. Jan. 1931).

rat unit and 1 female frog unit is comparable to $\frac{1}{2}$ rat unit when testing for pituitary gonadtropins.

For the recognition of certain diseases, particularly malignant tumors of the testicle and chorionepithelioma following pregnancy, a quantitative evaluation of the increase in chorionic gonadotropins is desirable. A quantitative technic using the Aschheim-Zondek reaction has proven very effective in our laboratory. Groups of 3 immature mice are injected with varying amounts of urine which may either be concentrated or diluted, and thus a wide range of hormone levels can be determined.

The right half of Table 16.2 contains data which permit the assay of chorionic gonadotropins in the urine at levels ranging from 100 to 1,000,000 M.U. per 1000 ml. urine, which has proven to us sufficient for most cases.

THE FEMALE SEX HORMONE

General Considerations. The female sex hormones comprise a group of chemically related compounds which are produced in humans principally by the ovaries and the placenta. The three most important naturally occurring estrogens are alpha estradiol, estrone, and estriol (Riley). They are produced in the non-pregnant female principally by the follicular cells of the ovary under stimulation of the pituitary and secreted into the blood stream. They produce certain effects upon the tissues of the female leading to the development and maturation of the internal and external female genitalia, the changes inherent to the menstrual cycle and to the development of certain features in the female organism characteristic of its sex (hair distribution, breasts, psychic attitudes). They are metabolized by the liver and excreted in the urine.

The quantitative determination of the female sex hormones is important in the proper evaluation of endocrine disorders in the female, especially disturbances in the menstruation. They can be determined by direct and indirect methods of assay. The former is more precise but much more complicated and is only used in larger clinics. Since the amount circulating in the blood is usually small except in the later stages of pregnancy, the determination of estrogens in the 24-hour urine sample is usually the method of choice. The indirect evaluation by means of the vaginal smear and the endometrial biopsy presupposes a normal female genital tract, and while much easier is less accurate.

Direct Methods of Assay. The determination of estrogenic hormones in human tissues and tissue fluids has been much better standardized since their chemical structures have become known and crystalline pure

substances could be used for the determination of standards. For the direct assay of estrogens in the blood and urine their recovery in concentrated and relatively pure form is essential. This can be accomplished in the blood by mixing 40 ml. of venous blood with 30 gm. of powdered anhydrous sodium sulfate and extracting the pulverized blood cake with 95 per cent alcohol. With this method a positive reaction should be obtained regularly 21 days before the onset of the menses, while one-half to one-fourth of the extract should elicit a positive reaction 7 days preceding the menstruation. The extraction of estrogens from the urine can be best accomplished by extracting the boiled and acidified 24-hour specimen of urine with benzene and further purifying the extract according to the method of Callow and associates. We have successfully substituted the extraction by the cumbersome Gallagher-Koch extraction apparatus with shaking the urine for 30 minutes with the solvent. The extract is then evaporated to oily consistency and taken up in a measured quantity of propylene glycol. The total amounts of estrogens for a 24-hour urine specimen can then be computed from the figures of Table 16.3

The most widely used method of biological assay is the determination of the effect of the isolated estrogens upon the vaginal epithelium of ovariectomized animals (Allen-Doisy method). The Allen-Doisy Rat Unit was originally defined as that quantity of material which would produce a cornified vaginal smear in castrated rats weighing 140 gm. plus or minus 20 gm. For the purposes of more accurate comparison the International Unit has been established, which is defined as the

TABLE 16.3

Table Showing the Animal Units Obtained with Various Doses and Dilutions of Urine Extracts

Interpretation of results in animal units per 24 hour specimen as indicated by positive reaction in 100 to 75 per cent of test animals used.

Amount Injected into 1 Animal	Dilution of 24-hour Urine Extract to ml.:					
	30	20	15	10	6	3
ml.						
1.5	20	13	10	6	4	2
1.2	25	17	12	8	5	2.5
0.9	33	22	16	11	6.5	3.3
0.6	50	33	25	17	10	5
0.3	100	66	50	33	20	10
0.15	200	130	100	66	40	20

effect of 0.1 microgram of crystalline estrone. One Rat Unit is approximately 10 International Units.

The spaying of the assay animals (rat or mouse) is best performed in weanling immature females (Emmens). In order to assure complete ovariectomy the ovary with the Fallopian tube should be resected at the uterine horn. Complete castration can be easily performed through a single transverse incision across the midline in the back of the animal and the wound can usually be closed by a single skin suture. Before using a group of spayed animals it is essential that their sensitivity to the estrogen be increased by a single primary injection of 0.5 to 1 gamma of estrone or diethyl stilbesterol. For the actual performance of the test numerous variations and modifications of the Allen-Doisy test are available. It is safe to state that those modifications which tend to give the highest degree of sensitivity are also the least accurate ones, and the combination of high sensitivity and high accuracy is still to be achieved. The substance to be assayed is usually taken up in propylene glycol, olive oil or sometimes an aqueous solution is prepared. It is usually administered parenterally in 2 to 4 injections over two days. Vaginal smears are taken on the afternoon and evening of the third and the morning of the fourth test. Smears may be taken with a fine metal spatula, cotton wool swabs, or by means of a glass pipette, and they may be examined unstained or stained with methylene blue, Papanicolaou or Shorr's method. A positive smear contains nucleated or cornified epithelial cells but no leucocytes. The response is considered positive if any of the three smears is positive. Many authors use a grading system for less complete vaginal changes and claim an increase in the sensitivity of the assay method. Fluhmann examines the mouse vagina histologically and can observe the appearance of mucified cells (3 plus reaction) with as little as $\frac{1}{32}$ of a Mouse Unit. The disadvantage of his method is of course the necessity of sacrificing the animals which otherwise can be used again after a rest period of three to four weeks and the establishment of a negative smear. Another method which promises to be sensitive as well as accurate uses the intravaginal administration of the assay material (Emmens).

Using immature non-spayed animals, Lauson and coworkers recommend the increase in the uterine weight as evidence of response to estrogens. We have obtained satisfactory results by using immature mice—similar to the assay of gonadotropins—and carefully weighing the blotted uterus on a Torsion scale. While this means of course also the sacrifice of the animal, it saves the spaying procedure and means that

for both gynecologically important bioassays essentially the same assay technic can be used. The important point in this technic is of course the strict separation of the estrogens from gonadotropins as well as from androgens and progestogens, since all these substances will lead to uterine weight increases.

Indirect Methods of Assay. Although theoretically less exact, indirect or clinical methods of assay for the presence of female sex hormones have gained so much in popularity that they have largely replaced the biological methods of assay. The reason for this is chiefly the great simplicity of clinical assays which makes the test suitable for an office procedure. Utilizing the mucous membranes of the female genital tract, the indirect methods of assay are based upon changes observed in the vaginal mucosa and the endometrium.

Samples of the vaginal epithelium are obtained either by the suction or the scraping method. The smears are stained with hematoxylin-eosin (Papanicolaou and Shorr), scarlet orange and fast green (Shorr) or Masson's trichrome, dehydrated and mounted under balsam. The absence of squamous epithelium and the presence of leukocytes and bacteria are diagnostic for estrogenic deficiency. Estrogenic deficiency can also be diagnosed when the atrophic cells are interspersed with squamous epithelial cells but still are the predominant cell type in the smear. In moderate estrogenic deficiency the majority of cells are of the squamous cell variety, staining either basic or acid. The nuclei are either large and vesicular or small and pyknotic. In conditions of estrogen saturation of the vaginal mucosa the squamous cells stain strongly acid, the cytoplasm appears keratinized, the nucleus extremely small, fragmented or entirely absent. The presence of progestin is recognized by signs of cell sloughing with wrinkling of the keratinized cytoplasm and folding of the edges.

In the hands of a man with sufficient experience the interpretation of the vaginal smear has become as nearly conclusive as the methods of direct hormonal assays. However, from our own experience we would warn inexperienced clinicians against relying too strongly upon their own interpretations of vaginal smears. To us the vaginal smear has proven its greatest value in the differential diagnosis of amenorrheal states. Here amenorrhea with maintenance of estrus (follicular cysts, granulosa cell tumor, pregnancy) can be well differentiated from the hypo-ovarian types (von Haam).

Another method of indirect assay is represented by the endometrial biopsy. It can be performed as an office procedure and in the hands of

an experienced pathologist is a very accurate means of evaluating the status of ovarian function. The proliferative or pre-ovulatory phase of the endometrium is characterized by narrow and long endometrial glands formed by high columnar epithelial cells showing a dense homogeneous cytoplasm. Glandular and stromal mitoses are frequent. Immediately after ovulation has occurred the beginning of the secretory or progestational phase of the menstrual cycle can be recognized by the appearance of basal vacuoles in the glandular epithelium and edema in the endometrial stroma. As the luteinization of the ruptured follicles progresses the secretory vacuoles fill the entire cells of the endometrial glands, which now become dilated and assume corkscrew-like aspect. The stromal cells swell with glycogen and the superficial layers of the endometrium assume a pseudodecidual appearance. The onset of the short menstrual phase of the endometrium is characterized by the appearance of leukocytes in the stroma and fragmentation of endometrial glands, which now are lined by a low cuboidal epithelium.

In the menstruating woman it is necessary to know exactly the day of the cycle on which the biopsy has been taken in order to permit the proper correlation. In our own experience endometrial biopsy has proved most conclusive if taken two or three days before expected onset of menstruation. At this time it will permit definite conclusions with regard to the amounts of estrogen which have been secreted during this cycle and the presence or absence of corpus luteum formation (von Haam).

THE CORPUS LUTEUM HORMONE

General Considerations. The corpus luteum is now established as an endocrine gland the primary function of which is the preparation of the uterine endometrium for implantation of the fertilzed egg. Its hormone has been successfully isolated and crystallized and its physiological properties investigated. The classic method of bio-assay has been described by Corner and Allen and consists in the histological demonstration of luteinized endometrium in sexually mature rabbits isolated for about 3 weeks and castrated 18 hours after mating. Although this method is a very reliable means of determination, it is too long and tedious to use as a diagnostic procedure. As one of the chemically related compounds, pregnanediol has been isolated by Marrian, and proof has been furnished that it apparently originates from the corpus luteum hormone—progesterone. It was found that four factors are concerned in the transversion of progesterone to sodium pregnanediol glucuronidate: ovarian, endometrial, hepatic, and renal.

A normal woman excretes from 5 to 24 mg. per day during the corpus luteum phase of the normal cycle and as high as 40 to 50 mg. per liter of urine have been found in pregnancy. It practically disappears within twenty-four hours after delivery. Although the data for pregnanediol excretions are given for a twenty-four hour sample of urine, it is necessary in the studies of nongravid patients to extract a forty-eight hour specimen to obtain a determinable quantity. In pregnant patients a twenty-four hour specimen is sufficient.

The oldest and still probably the most accurate method for the determination of pregnanediol excretion in the urine is the method of Venning published in 1937 and 1938. A 24- or 48-hour sample of urine is extracted several times with n-butanol and the combined extracts are precipitated with acetone. It is then purified by dissolving it in water and reprecipitating it with acetone. Finally it is dissolved in hot ethanol and the solution evaporated to dryness. The weight of the residue and its melting point are obtained. This method, which determines the total amount of excreted free and conjugated pregnanediol as sodium pregnanediol glucuronidate, is not easy and numerous attempts have been made to substitute a simpler technic which would lend itself better to routine clinical laboratory procedure. Astwood and Jones liberated pregnanediol by hydrolizing the urine with hydrochloric acid, and Talbot and coworkers applied to this "free pregnanediol" the yellow color reaction obtained by treating the precipitate with concentrated sulfuric acid. Haslewood recommends the method of Sommerville, Marrian and Kellar, which is based on the Astwood-Talbot principle and which permits a rapid determination of pregnanediol with fair accuracy.

Test for Pregnancy. The excretion of pregnanediol has been suggested as a chemical test for pregnancy by Guterman. A qualitative color reaction of a "deep yellow" produced by sulphuric acid with the precipitated purified and isolated pregnanediol has been regarded as positive evidence for pregnancy in women "who have missed at least one menstrual period." Our own experience with this test has been published by Reinhart and Barnes and agrees with the many other unfavorable reports regarding the value of the Guterman test in the diagnosis of pregnancy or threatened abortion.

THE MALE SEX HORMONE

The Urinary 17-Ketosteroids

General Considerations. The 17-ketosteroids are steroids possessing a ketone group on the 17th carbon atom and are in principle the urinary excretion products of the androgenic hormones of the testis and the

adrenal cortex (Selye). Their concentration in the urine reflects fairly accurately the androgenic activity of those two endocrine glands and their chemical determination has proven more accurate and more practical for clinical purposes than the previously recommended bioassay of androgens by means of the comb of the capon or the baby chick (Gallagher and Koch, Dorfman). The 17-ketosteroids can be subdivided by digitonin precipitation into the normally predominating alpha and the less important beta fraction, but they are usually determined as total 17-ketosteroids and their excretion is expressed in mg. per 24 hour urine sample. Children under 6 years excrete usually less than 1 mg. regardless of sex, adult males excrete 7 to 20 mg., while adult females excrete from 5 to 15 mg. during 24 hours (Rakoff). It has been estimated that 30 to 40 per cent of the 17-ketosteroids excreted by men are derived from the testes and 60 to 70 per cent from the adrenal, while the 17-ketosteroids excreted by women are nearly all derived from the adrenal glands. With advancing age the 17-ketosteroid excretion slowly decreases. Abnormally low 17-ketosteroid values are found in cases of severe hypopituitarism, Addison's disease and hypothyroidism as well as in conditions of general malnutrition and during or after severe illness. In men with hypogonadism or after castration the 17-ketosteroid excretion is usually only moderately decreased. Abnormally high values for 17-ketosteroids (over 100 mg.) are found in men with interstitial cell tumors of the testicle, and in men or women with hyperadrenalism either due to a cortical adrenal tumor or due to pituitary hyperadrenalism (cortical hyperplasia).

Collection of Urine Sample. Engel emphasizes that the collection of a complete 24-hour specimen of urine often represents the most difficult task of the entire assay procedure. The first morning specimen should be discarded and then all subsequent samples including the first specimen on the next morning should be collected in a large glass bottle which contains 5 ml. of toluene as preservative. If the method of collection is not very reliable the measurement of the creatinine excretion is recommended as additional check for the correctness of the collection.

Method of Assay. The principles of the determination of 17-ketosteroids in the urine consist of: freeing the steroids from their conjugate by acid hydrolysis, extracting and purifying them from the acidic and phenolic materials and the urinary pigments, and subjecting them to colorimetric analysis by utilizing the Zimmermann reaction. Of the many various modifications available we have found the method of Drekter and coworkers practical, rapid and sufficiently accurate for

clinical purposes. The advantage of their method is the avoiding of the costly reflux apparatus and the employment of small aliquots of the 24-hour urine sample; the disadvantage is of course the magnification of any error in the assay. Since, however, only large deviations from normal values are clinically significant, minute accuracy is not as essential as in other quantitative tests. The following is the outline of the method practiced now in our laboratory.

Extractions:

1. Add 3.0 ml. of concentrated hydrochloric acid to 10 ml. of urine taken from the 24 hour specimen.
2. Place in water bath between 80 and 100°C. for 10 minutes.
3. Cool rapidly
4. Transfer urine with 2 or 3 washings with distilled water to a 125-ml. separatory funnel and shake vigorously for 1 minute with 20 ml. of C.P. anhydrous ethyl ether.
5. Allow layers to separate and discard urine.
6. Wash ether extract with 10 ml. of 10 per cent NaOH and twice with 10 ml. of distilled water.
7. Evaporate ether to dryness in small Erlenmeyer flask using mild heat. The residue can be saved in the refrigerator for a later assay.

Colorimetric Procedure:

1. Dissolve ether residue to 2 ml. of absolute alcohol.
2. Place 0.2 ml. of this solution in colorimeter tube.
3. Add 0.2 ml. of the m-dinitrobenzene reagent.
4. Add 0.3 ml. of 2.5 N alcoholic KOH solution.
5. Incubate at 27°C. for 90 minutes in the dark.
6. Add 95 per cent alcohol to the 10-ml. mark.
7. Read in the colorimeter using a 540 filter.
8. Prepare standard solution for comparison:
   0.20 ml. 17-ketosteroid standard
   0.20 ml. m-dinitrobenzene reagent
   0.30 ml. alcoholic KOH
9. Prepare reagent control for correction:
   0.20 ml. absolute alcohol
   0.20 ml. m-dinitrobenzene reagent
   0.30 ml. alcoholic KOH

   Run standard and reagent blank simultaneously with unknown and subtract reading of reagent blank from reading of standard and unknown.
10. The concentration of urinary 17-ketosteroids for 24 hours in mg. is then obtained according to the formula (Engel):

$$\text{Mg. 17-ketosteroids/24 hr.} = \frac{\text{O.D.E. Cor.}}{\text{O.D. Std.}} \times \text{mg. dehydroisoandrosterone in standard tube} \times \frac{\text{total vol. extract}}{\text{vol. extract analyzed}} \times \frac{\text{total vol. urine}}{\text{vol. urine hydrolyzed}}$$

which in our case can be simplified to:

$$\frac{\text{O.D.E. cor.}}{\text{O.D. Std.}} - \frac{2 \times \text{total vol. urine}}{100}$$

O.D.E. means optical density of extract, O.D. Std. optical density value of standard.

Preparation of Reagents.

1. *M-dinitrobenzene Reagent.* Dissolve 20.0 gm. of commercially available m-dinitrobenzene in 750 ml. of 95 per cent ethyl alcohol. Heat to 40°C. in the water bath and add 100 ml. of 2N NaOH. Cool for 5 min. and add 2500 ml. of distilled water. Filter off precipitate in Buchner funnel and wash several times with distilled water. Dry on filter paper using suction. Recrystallize twice, once from 120 ml. of hot absolute alcohol and once from 80 ml. of hot absolute alcohol (melting point 90 to 91°C.). Prepare 1.15 per cent solution in absolute alcohol by dissolving 34.5 mg. of purified crystals in 3 ml. of absolute alcohol. Use solution for not more than 1 week.

2. *Alcoholic KOH Solution.* Dissolve 10 gm. of C.P. KOH in 50 ml. of ice-cold absolute alcohol. Centrifuge and titrate supernatant against 0.10 N HCl using methyl orange as indicator. Adjust to 2.5 N with absolute alcohol. Keep cold all the time and do not use it if any color is present.

3. *17-Ketosteroid Standard.* Dissolve dehydroisoandrosterone in absolute alcohol so that 100 ml. contains 10 mg.

Urinary Corticoids

Urinary corticosteroids can be defined as "urinary substances which possess the typical 21-carbon-atom skeleton of the adrenal steroids and at least one other structural detail which is characteristic of those steroids" (Mason). Pincus in a recent survey grouped the methods of corticoid assays which have been investigated more in detail into four: 1) measurement of reducing activity of corticosteroid extracts (Heard and Sobel, Talbot and coworkers); 2) measurement as formaldehydogenic substances (Daughaday and coworkers); 3) measurement as tetrazolium-reacting substances (Chen and coworkers); 4) as chromogen in the 17-hydroxycorticoid method of Porter and Silber. Only a small amount of corticosteroid appears free in the urine, while the bulk of the hormones is excreted as conjugates of glucuronic acid. Since the corticoids are easily destroyed by strong acids or alkalis the problem of freeing them by hydrolysis is quite difficult. Mason concluded that

extraction at pH 1 using heat not in excess of 50°C. is adequate for most clinical purposes. For exact work enzymatic hydrolysis using spleen beta glucoronidase has been suggested (Dustan and coworkers). Bioassay methods for urinary corticoids are more specific but too time-consuming to be used in routine clinical and hospital work (Paschkis). The great uncertainty which still exists with regard to optimal conditions for hydrolysis make it impossible to carry out quantitative balance or recovery studies.

Complete abandonment of the hydrolysis of urinary corticoids and the introduction of butanol extraction have led recently to new methods which may well revolutionize the entire field of corticoid assays (Wolfson). Reddy, Jenkins and Thorn have combined butanol extraction with the method of Porter and Silber and thus devised a technic which appears simple and accurate for the estimation of 17-hydroxycorticoids in urine. In their hands the method has demonstrated a good correlation with known states of adrenal function and they recommend it as a rapid and convenient method for estimating total 17-hydroxycorticoid excretion during adrenal activation or the administration of adrenal cortical hormones.

Procedure:

1. A 24-hour specimen of urine is collected in the cold (0 to 4°C.) without preservative and stored in the frozen state until extraction.
2. 5 to 20 ml. of the specimen are adjusted to pH 1.0 by dropwise addition of 50 per cent sulfuric acid.
3. Two successive extractions are carried out with the use of 0.5 volume n-butanol shaking a minimum of 3 minutes each time. Emulsions are readily dissolved by centrifugation.
4. The combined extracts are washed with 0.2 volume of 0.1 N sodium hydroxide and 0.2 volume of 0.01 N sulfuric acid. Each wash is shaken one minute only. The extracts are then ready for colorimetry.
5. 4 ml. of phenylhydrazine-sulfuric acid reagent is added to:
   1 ml. butanol extract (Sample A)
   1 ml. butanol (Blank A)
   1 ml. cortisone standard (Standard A)
6. 4 ml. of dilute sulfuric acid reagent is added to:
   1 ml. butanol extract (Sample B)
   1 ml. butanol (Blank B)
   1 ml. cortisone standard (Standard B)
7. The tubes are thoroughly mixed and incubated in a constant temperature water bath at 60°C. for exactly 20 minutes and transferred to a cold water bath for 5 minutes.
8. The color is read at 410 millicrons in a spectrophotometer (Coleman Junior Model 6A) set at zero with distilled water.

9. Calculation:

$$\text{Sample A} - \text{Sample B} = a$$
$$\text{Blank A} - \text{Blank B} = b$$

a–b = corrected optical density (C.O.D.) of unknown sample. The C.O.D. for the standard is derived in the same manner.

$$\text{mg./24 hrs. 17-hydroxycorticoid} = \frac{\mu\text{g./ml. standard}}{\text{C.O.D. standard}} \times \text{C.O.D. sample} \times \text{24 hr. urine volume (ml.)}$$

Reagents:

1. n-Butanol, reagent grade, Merck.

2. Dilute sulfuric acid reagent: Dilute 620 ml. concentrated sulfuric acid to 1000 ml. in distilled water.

3. Phenylhydrazine-sulfuric acid reagent: Dissolve 65 mg. phenylhydrazine hydrochloride in 10 ml. of dilute sulfuric acid. The solution is stable for one week at 0 to 4°C.

4. Cortisone standard: Prepare solutions containing 10 and 20 microgram per ml. by dissolving cortisone (free alcohol or cortisone acetate) in butanol.

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CHAPTER 17

EXAMINATION OF TRANSUDATES, EXUDATES, SKIN AND MUCOUS MEMBRANES

By Emmerich von Haam

Transudates and Exudates

GENERAL CONSIDERATIONS

Normally serous cavities contain a certain small amount of fluid which is kept in balance by the osmotic pressure within the capillaries of the serous membranes. A decrease of the colloid osmotic pressure of the blood, an increase of the intracapillary blood pressure, or damage to the capillary endothelium may produce an accumulation of this fluid in serous cavities in excessive amounts. This fluid is non-inflammatory in origin and is known as transudate. It is observed in conditions like nephrosis, cardiac decompensation, obstruction of the venous circulation, or certain toxic conditions and tends to recur unless the causal factor is removed. Depending upon its location it is known under different terms, such as hydrothorax, hydropericardium, ascites, hydroarthrosis.

On the other hand, exudates are produced as the result of inflammatory conditions brought about by local injury to the tissues. They are usually the consequence of acute or chronic infectious processes but may be also produced by mechanical and chemical irritants. They always carry an increased number of cellular elements, red or white blood corpuscles and endothelial cells, and differ from transudates also in many important physicochemical and chemical aspects. From the serous membrane emitting the exudate in the course of an inflammatory involvement of that membrane, it is termed exudative pleurisy, pericarditis, peritonitis, arthritis, etc. Often the adjective "serous," "fibrinous," "hemorrhagic," or "purulent" is added dependent upon the character of the exudate. Some disease processes, such as tuberculosis, are characterized by a rather typical appearance of the exudate, while others produce nonspecific exudate processes. In contrast to transudates, removal of exudates often has a definitely beneficial influence upon the causative disease process and often the exudate will not recur. This different therapeutic response is an additional reason why a differentiation between the two types of fluid accumulation is of great importance.

OBTAINING PUNCTURE FLUIDS

Samples of transudates and exudates can usually be readily obtained for laboratory examination by paracentesis. Certain principles should be borne in mind in the collection of these effusions. It is usually difficult to determine before the fluid is examined in the laboratory whether it is a transudate or exudate, so the utmost precautions should be taken to see that the fluid obtained is kept uncontaminated and not allowed to coagulate since coagulation usually occurs in exudates. There should be proper sterile containers, the size depending upon the amount of fluid expected to be removed. Sterile sodium citrate solution (2.5 per cent) should be added in proportion of 10 ml. to 100 ml. of effusion. If possible, 100 ml. or more of the fluid should be sent to the laboratory for examination. The usual methods of sterilization of instruments and glassware should be employed. Aseptic technic is used and the fluid removed under local anesthesia.

Fluid from the abdominal cavity is obtained by the process of paracentesis abdominis. The patient should be in sitting position with his back well supported by a backrest or pillows. The bladder should be emptied before the puncture is made. A needle or cannula of 16–18 gauge should be used. The best point of puncture is in the midline usually about half way between the symphysis pubis and the umbilicus. It is the least likely point to encounter loops of intestines fixed by adhesions. When a large amount of fluid is to be withdrawn, it is advisable not to allow the fluid to drain rapidly and to put on an abdominal binder afterward.

Fluid from the pleural cavity is obtained by thoracentesis (paracentesis thoracis). The patient sits upright or has the shoulders elevated on pillows, leaning forward with the arms raised and his hands on the opposite shoulders. The area of greatest dullness is determined. If the point selected is in the sixth or seventh interspace, the puncture should be in the mid-axillary line; if in the eighth interspace it should be below the angle of the scapula. The puncture should be made close to the upper border of the rib to avoid the intercostal blood vessels. The fluid is best obtained by aspiration into a syringe with a 16 gauge needle or into an aspirator bottle. Fluid should not be withdrawn too rapidly since sudden pulmonary edema or a rapid fall in blood pressure may develop.

Fluid from the pericardium is obtained by paracentesis pericardii. The patient is kept placed in a reclining position and the needle puncture placed in the fifth interspace below the left nipple with the shaft of the

needle directed upward and medially. Sufficient fluid for examination can be obtained with a 10-ml. syringe attached to a 20-gauge needle. If the needle touches the heart and puncture of the myocardium is imminent, pulse-like movements of the instrument can be observed. Large pericardial effusions should be removed by pericardotomy.

Fluid from the joint cavities can be readily obtained with little pain and discomfort. The joint is placed in resting position and after liberal infiltration of the skin and deeper tissues with novocaine the joint is punctured at the most fluctuant point of swelling. It must be remembered that periarthritic edema often increases considerably the volume of the diseased joint and that even markedly swollen joints often contain only a small amount of exudate. For joint punctures a short needle should be used always and care taken not to traumatize unnecessarily the synovial membrane or to injure the joint cartilage.

Hydrocele fluid is obtained from the scrotal sac by the introduction of the needle into the anterior surface taking care that the testis and epididymis are avoided.

The fluid content of cysts or vascular formations (blood or lymph vessels) may occasionally require a diagnostic examination. Unless the pathology of the tissue to be punctured is exactly known it is recommended that a very fine needle be used for the first puncture in order not to injure the wall of the cystic structure too much. Sufficient fluid must be withdrawn at the first puncture to decrease the fluid pressure enough to prevent seeping. Should the cystic structure prove to be an aneurism a pressure bandage applied immediately will prevent further oozing if the puncture wound is not too large.

Fluid accumulations in non-preformed cavities are observed in generalized or localized edema, hematomas, and exudative cellulitis, diffuse or with abscess formation. It is usually only possible to obtain small amounts of interstitial fluid for bacteriological or chemical examination with the exception of pus in large abscesses. In obtaining pus from an abscess one must take care not to infect the healthy tissue surrounding the abscess or open up blood channels to the infectious process. Because of the thickness of the pus a 20-gauge needle is recommended.

PHYSICAL CHARACTERISTICS

The *quantity of the fluid* to be examined should always be determined. If it represents the total amount present it will give valuable information concerning the extent of the pathological process. If it is only a representative sample, the quantity will give us an index of the accuracy of

the various laboratory tests requested. With the exception of the cytological methods, more accurate results are obtained with larger test samples and not less than 100 ml. should be sent to the laboratory if large quantities of fluid are available.

The *color* of the fluid is of considerable diagnostic importance. Fluid containing only plasma or serum will be light to dark yellow dependent upon the bilirubin content of the plasma. The admixture of red blood cells will give the fluid a reddish tinge to a dark red or reddish brown color. The admixture of white blood cells will give the fluid a white to yellow color, chyle will tinge the fluid a milky green. Various bacterial infections are prone to give exudates characteristic colors; tuberculous exudates are grayish, exudates infected with pyocyaneous are bluish green, putrid changes will discolor hemorrhagic fluids and give a deep brownish black color.

The *turbidity* of the puncture fluid depends upon the amount of suspended formed particles, mostly cells. White cells will produce all degrees of turbidity from a slight cloudiness to thick creamy pus. Red cells will cause a grayish red turbidity, fat droplets (chyle) will produce a milky turbidity. Prolonged standing will cause sedimentation and the formation of layers with various degrees of turbidity.

Coaguability of puncture fluid depends upon the amount of fibrin suspended in the fluid. It represents an important point of differentiation between exudates and transudates. The latter rarely show spontaneous complete or partial coagulation. Notable exceptions are chyliferous transudates or those occurring during neoplastic processes. Exudates, on the other hand, coagulate usually very quickly after removal from the serous cavity. Only if the fibrin has been destroyed by bacterial or cellular enzymes, as observed in certain purulent processes, do exudates lose the ability to coagulate. Coagulation of puncture fluid may be prevented by the addition of sterile sodium citrate to the freshly obtained fluid.

The *odor* of the puncture fluid has little diagnostic importance. Exudates and transudates are usually odorless unless combined with putrefactive changes. Certain bacterial infections tend to produce a sweetish odor.

The *specific gravity* of puncture fluids is of utmost importance in the differentiation of transudates from exudates. If sufficient quantities of fluid are available (25 ml. or more) a simple Squibb urinometer may be used. If only small quantities are available Exton's immiscible balance is recommended. It requires only a few drops of puncture fluid

and gives reliable results. For accurate results it is important that the readings are corrected for temperature. Transudates usually show a specific gravity varying between 1.006 and 1.018, while exudates, due to their greater content of protein (over 3 per cent), have a specific gravity over 1.020. Occasionally transudates accompanying neoplastic processes or occurring in diabetic patients show a higher specific gravity (up to 1.025). The admixture of blood during the process of puncture may influence the specific gravity of the fluid obtained and has to be considered in the diagnostic evaluation.

By carefully checking the physical qualities of the puncture fluid it will usually be possible to accurately classify the type of fluid. Not only can the general differentiation between transudate and exudate be made but also the type of exudate can usually be recognized. Thus an accurate evaluation of the physical characteristics gives us important leads as to the additional tests to be employed for the accurate diagnosis of the causative factors involved in the pathological accumulation of the fluid.

CHEMICAL EXAMINATION

Protein. The content of protein in puncture fluids varies from 0.1 to 8 gm. per 100 ml. Transudates usually show a low content of protein unless a reabsorption of the fluid has taken place and has led to a concentration of solids. Rarely are values higher than 2.5 gm. per 100 ml. of fluid. Most of the proteins consist of albumin with some mucin-like substance appearing in fluids from joints. Exudates show a marked increase in protein, especially the globulin fraction in the cell-free portion of the fluid. It will approach the plasma protein concentration and also show an increased amount of fibrinogen. Values ranging from 3 to 8 gm. per 100 ml. of fluid are common in all acute exudates. Inflammatory processes of more chronic character, such as tuberculosis, tend to give lower protein values (about 1.5 to 2 gm.). The following methods are used for the determination of total protein in puncture fluids.

Rivalta's Qualitative Test

Place 200 cc. of water in a cylinder. Add two drops of glacial acetic acid and mix. Allow two or three drops of the fluid to be examined to fall into the solution. If the fluid is an exudate a distinct cloud is observed in the wake of the falling drop, while in the case of a transudate no such turbidity is seen.

Methods which determine quantitatively the protein content of body fluids are Esbach's quantitative method and the Shevky-Stafford modification of Tsuchiya's method.

Glucose. Determined with the same methods as in the blood, the glucose content of transudates is usually the same as in the plasma. Exudates show a lower glucose content due to the glycolytic activities of bacteria and inflammatory cells.

Lipids. Neutral fats and fatty acids are usually not present in transudates. Only the admixture of chyle will increase the lipid content of transudates. In exudates the capillaries are permeable for lipids, and fat and cholesterol are found in exudative tuberculous processes.

Minerals. Sodium chlorides are usually higher in transudates and about the same as in the blood in exudates. The levels of calcium, magnesium and potassium are slightly higher in exudates than in transudates due to the increase in protein. The content of phosphorus is equal to that of the blood.

The methods of determining these in puncture fluid are identical with those of blood chemistry. The practical value of their determination is negligible.

BACTERIOLOGIC EXAMINATION

Direct Examination. It is seldom necessary to examine transudates for bacteria, but with all fluids suspected of being exudates it is essential that centrifuged samples of the fluid be examined for bacteria. Some exudates, especially when markedly purulent, may be examined directly by placing some of the fluid on a slide, allowing it to dry, fixing it by heat or with methyl alcohol and using the customary bacterial stains. Smears stained with Wright's stain do not require previous fixation and all bacteria will be dark blue while the cells of the exudate will stain in the customary fashion.

Cultural Methods and Animal Inoculation. These methods are of great value in the recognition and differentiation of the various bacteria and fungi. Animal inoculation is of particular value in establishing the presence of tubercle bacilli (*Mycobacterium tuberculosis*). The only disadvantage of this method is that usually four to six weeks must elapse before the results are known.

For details of these methods the student is referred to Chap. 12 on Medical Bacteriology.

CYTOLOGIC EXAMINATION

Cytologic examination of puncture fluids is of extreme importance and should be done in every case. The cells found in transudates are few in number and consist chiefly of endothelial cells with an occasional

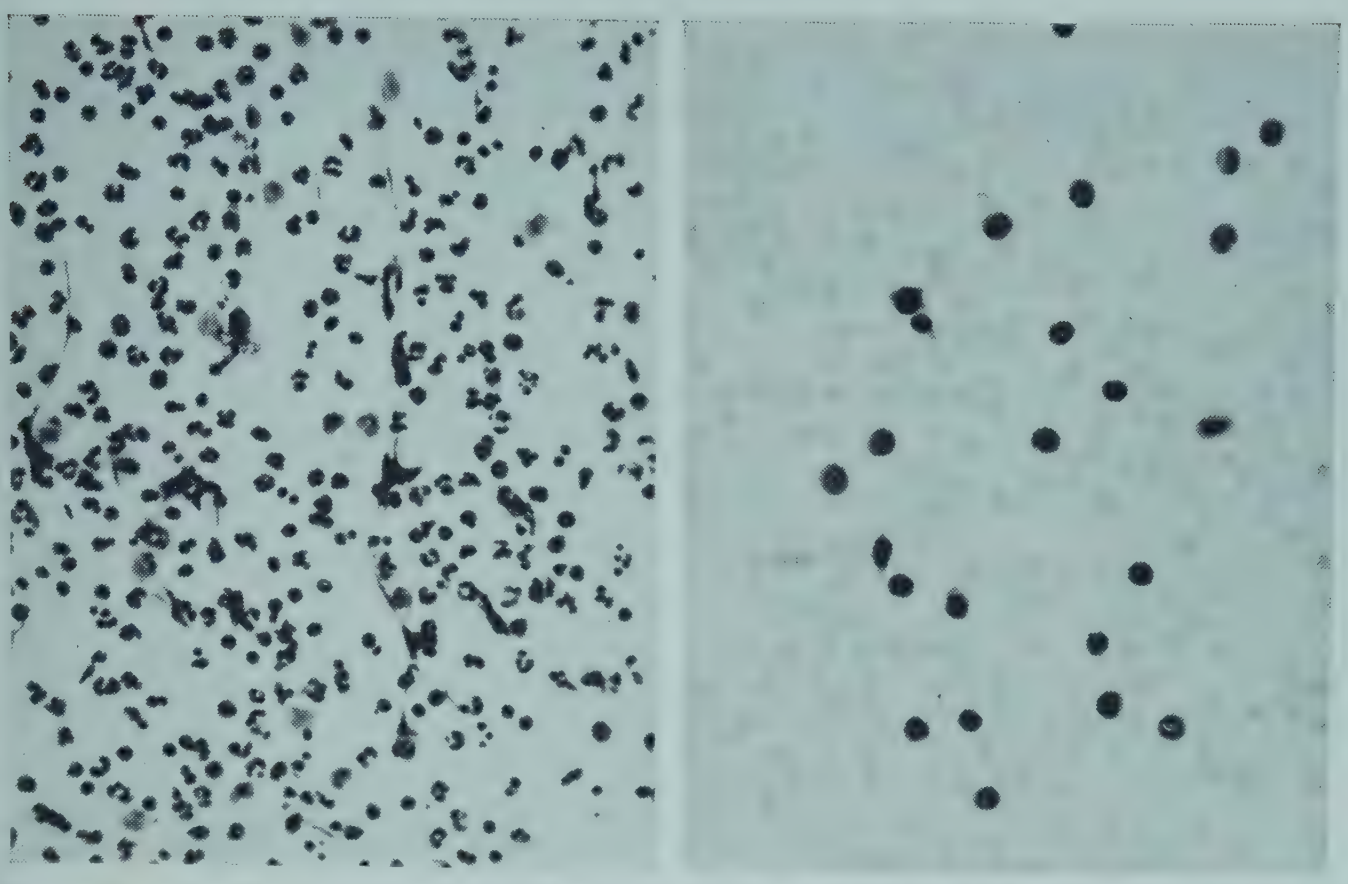


FIG. 17.1. Left: Purulent exudate of pleural cavity in empyema (Papanicolaou, ×370). Right: Chronic hemorrhagic exudate of pleural cavity in tuberculosis (Papanicolaou, ×370).

lymphocyte and rarely neutrophil leukocytes. Exudates usually contain large numbers of cells with neutrophil leukocytes the predominant type (Fig. 17.1). A correct cytological examination will give important information with regard to the nature of the inflammatory process, the amount of injury exerted by the infectious agent, and the local immune biological processes. Neoplastic processes can be recognized by the appearance of tumor cells.

Total Cell Count. The estimation of the total number of cells in puncture fluids is of little practical value. The cells may be counted by the same technic as employed in counting the leukocytes of the blood, and total counts may show as few as 100 cells per cu. mm. in transudates and over 50,000 per cu. mm. in purulent exudates. Cell counts should always be performed on the non-centrifuged and non-coagulated specimen after thorough shaking to assure an even distribution of cells.

Differential Count. For a differential study of cells in puncture fluids smears should be prepared from the sediment obtained by centrifugation at high speed. In very turbid fluids this step may not be necessary and smears can be prepared without centrifugation. The slides are permitted to dry in the air and are stained with Wright's or Giemsa's stain, according to the method of staining blood smears. They are studied under oil immersion and a differential count of at least 100 cells is tabu-

lated. Neutrophil leukocytes are the predominating type of cell in all purulent exudates. The degree of cell maturation and the toxic changes of the cytoplasm and nucleus can be recognized in the smear. Lymphocytes predominate in chronic exudative processes, specifically tuberculosis. Lymphocytes are usually of the mature small cell variety and may show toxic granulations. Eosinophil leukocytes are present in exudates of allergic nature or accompanying parasite infections (Echinococcus). They are also found in certain types of tuberculosis, particularly after repeated pneumothorax treatment. Endothelial cells are found in all types of puncture fluids. They represent the predominating cell of transudates and are characterized by large cytoplasm, clear staining nucleus and evidence of phagocytic activity. They often appear in clumps.

In carcinomatosis of the serous surfaces, cells may be encountered, singly or in groups, which are large and irregular in size and which possess large dark staining nuclei. Sometimes abortive acinar or sheetlike formations can be noted. Mitotic figures are encountered not frequently since the majority of these cells are the result of desquamation. However, highly atypical mitotic figures can sometimes be demonstrated (Saphir).

Although most of the cellular changes can also be demonstrated on smears stained by the Wright or Gram method, we recommend the staining method of Papanicolaou for the clear demonstration of nuclear changes. In addition to a marked and disproportionate increase in size and a much denser chromatin network, the nuclei often possess very prominent, irregular and often multiple, nucleoli. These are sometimes triangular or bar-shaped, and if present, represent the most reliable characteristic of malignant cells (Fig. 17.2). We feel that a careful study of well prepared and well stained smears from pleural or peritoneal exudates usually permits the diagnosis of malignant processes with a much higher degree of accuracy than mentioned in the older texts. False negative results are encountered in cases where the cancer cells fail to desquamate, as in metastatic scirrhous carcinoma of the stomach. False positive diagnoses are usually made on the basis of clumps of degenerated serosal endothelial cells with pyknotic nuclei.

Histological Methods. It is recommended by Chapman and Whalen that the sediment be fixed, embedded in paraffin, sectioned and stained by histologic methods for demonstration of malignant cells. A sediment of at least 200 ml. of fluid should be packed tightly by centrifugation at high speed and fixed thoroughly with 10 per cent formalin. After fixa-

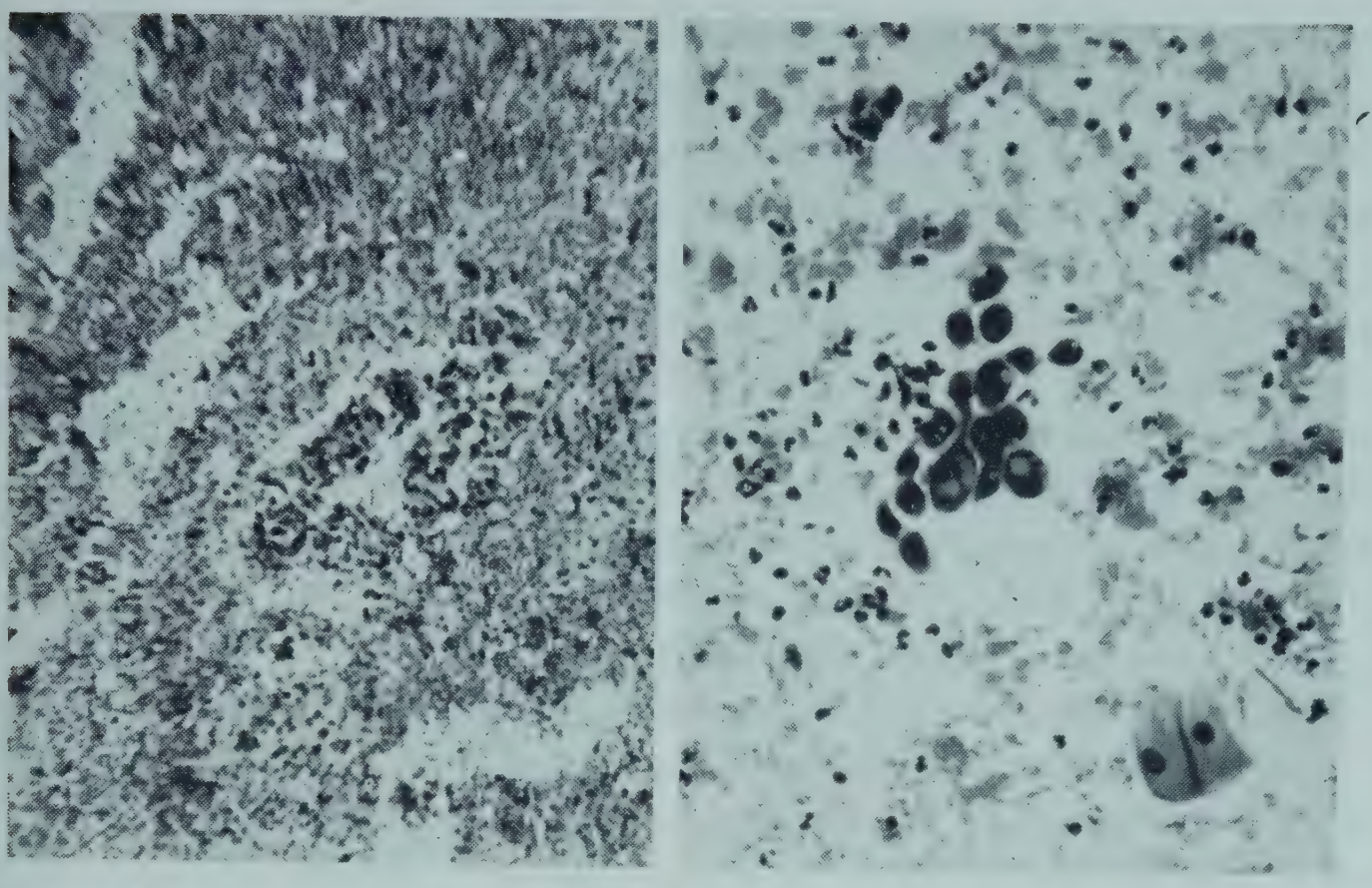


FIG. 17.2. Carcinomatosis peritonei from primary ovarian adenocarcinoma. Left: Cluster of malignant cells in cell block (H. & E., ×110). Right: Malignant cells in cell smear (Papanicolaou, ×200).

tion the minute clump of packed cells should be dehydrated with various concentrations of alcohol and imbedded in paraffin. The autotechnicon or any manual procedure may be used. The imbedded tissue can be sectioned with a microtome at 6 to 8 microns thickness and the sections stained with hematoxylin-eosin or other histological stains. The advantages of this method are that the cells will be concentrated and will have an appearance similar to that to which the pathologist has become accustomed by his histological methods. The pathologist then can more easily compare the size and shape of cells with those of established malignant processes and perhaps be more certain of his diagnosis. Of course, one of the most fundamental characteristics of malignancy, the invasive character of the malignant tissues, cannot be demonstrated. According to our experience the method is tedious and often the minute particles of tissue will be lost during the process. It offers no other advantage over the slide method except that of greater cell concentration.

SEROLOGIC EXAMINATION

Complement fixation tests on transudates and exudates may be performed for syphilis, gonorrhea, tuberculosis and echinococcus disease. Transudates are usually free of anticomplementary activity and may be

tested in the same manner as serum. As a general rule, however, the antibody contents is less than in serum and larger amounts of fluid are sometimes required than those employed in regular serological tests. For quantitative tests amounts of .5, .25, .125, .0625 and .03125 ml. of transudates are used. Exudates are much more likely to be anticomplementary and must be first tested for anticomplementary activity. They should be freshly collected and kept at low temperature until examined. Each should be heated for 15 minutes at 55°C. and tested for anticomplementary activity. The smallest amount of even slight interference of hemolysis represents the anticomplementary unit. In setting up the complement fixation test the usual dose of antigen is added to one-fourth of the anticomplementary unit, and the complement is titrated in the presence of the antigen in the usual manner (Kolmer).

For the diagnosis of echinococcus fluid, the fluid from echinococcic cysts preserved with 0.5 per cent phenol serves as antigen. Kolmer recommends preparation of the antigen by grinding up the moist scolices with fine sand and adding nine volumes of clear cyst fluid, thus giving an approximate 10 per cent extract of the scolices. Phenol may be added (2.25 per cent) and the mixture extracted in an incubator at 37°C. for four days, filtered and stored in a refrigerator.

CLINICAL CORRELATION

Pericardial Effusions. The normal amount of free fluid in the pericardial cavity is considerably greater than in other serous spaces. This serous fluid may be increased in amount in some cardiac and renal diseases, but the increase is not as large in amount as in the pleural and peritoneal cavities. A thick fibrinous plastic exudate may be seen in acute pericarditis of rheumatic fever. Purulent pericardial exudates are found most frequently in pneumococcic infections. In such cases the amount of effusion may be quite large. Pericardial effusions may be hemorrhagic in tuberculosis, malignant disease, or hemolytic streptococcic infection.

Pleural Effusions. A collection of fluid in the pleural cavities due to cardiac or renal diseases is called hydrothorax and has the characteristics of a transudate. In cardiac decompensation the fluid is more apt to collect in the right side though it may occur on both sides at the same time. In renal edema it is usually bilateral.

Serofibrinous effusions occur most frequently in pulmonary tuberculosis. Tubercle bacilli are usually absent in the centrifuged sediment, but often can be found by concentration methods or with animal inoculation.

The character of the fluid in empyema is determined by the causative bacteria. In pneumococcic infections the fluid is usually yellowish or greenish in color, thick and creamy. In streptococcic infections, usually of the hemolytic type, it is more seropurulent in character. In tuberculous empyema it may contain caseous masses in a rather thin purulent fluid. The pus from rupture of an abscess or gangrene of the lung is often putrid, having a foul odor.

Hemorrhagic fluids, particularly those of dark brownish appearance, strongly suggest malignancy in the lung. This appearance may also be found in fluids of pulmonary tuberculosis and rarely in other diseases. Chylous effusions are found in conditions where there is occlusion of the thoracic duct by trauma, malignancy, or filaria. Chyliform fluids, which are distinguished with difficulty from chylous fluids, are milky from the presence of fine fat droplets which in turn are probably the results of cell degeneration.

Malignant processes leading to pleural effusion are either primary bronchogenic carcinoma or metastatic tumors to the visceral or parietal pleura from the breast, gastro-intestinal or genitourinary tract. A careful cytological examination can often not only verify the presence of a malignant process but also give valuable clues as to its type and primary origin.

Peritoneal Effusions. These may be of the transudate type as found in cardiac or renal diseases, or they may be caused by interference with the portal circulation, as in cirrhosis of the liver, syphilis or carcinoma. This is called ascitic fluid and has the usual characteristics of transudates previously described.

In tuberculous peritonitis the fluid is usually thin and slightly cloudy, contains an increased protein content and has a specific gravity of above 1.020. Tubercle bacilli are rarely found in the fluid except by concentration methods and guinea pig inoculation. In peritonitis from colon bacillus infection the purulent fluid usually has a fecal odor. This characteristic is also noted after perforation of the intestine. In acute inflammatory conditions in which there has been no escape of intestinal organisms into the peritoneal cavity there may be a cloudy serous fluid, free of bacteria, containing small flakes of fibrin that may appear to be pus. This fluid, while having an increase in the total number of leucocytes, is not considered as purulent. Pus obtained from the abdominal cavity may show, in addition to degenerated leucocytes, numerous types of bacteria of which the colon bacillus is most common. The streptococcus, pneumococcus, staphylococcus and gonococcus may be found in occasional instances.

Peritoneal carcinomatosis may produce no fluid, a transudate or an exudate. Metastases from gastric carcinoma, even widespread over omentum or mesentery, usually fail to produce any recognizable amounts of fluid. Metastasis to the periportal lymph glands and liver from any malignant tumor may produce a transudate type of ascites with clear fluid and no malignant cells. Carcinomatosis from tumors of the ovaries usually produces a peritoneal exudate with an abundant number of malignant cells.

Synovial Fluid. The amount of synovial fluid obtained usually amounts to only a few cubic centimeters. The presence of numerous neutrophils characterizes a purulent arthritis. Bacteria may be found in acute purulent conditions either by smear or culture and tubercle bacilli by guinea pig inoculation. The organisms most frequently sought are gonococci, but they are found rarely on examination of direct smears. Cultures in reduced oxygen tension offer better results.

Skin and Mucous Membranes

GENERAL CONSIDERATIONS

Exudates on mucous membranes and skin are usually purulent in character. They are most frequently caused by an infectious agent the proper identification of which is the chief purpose of studying the exudate. For the various bacteriologic, parasitologic, mycologic and virologic methods used in the study of exudates on skin and mucous membranes the student is referred to the special chapters dealing with those subjects. It must be emphasized, however, that the causal factor of the exudative process cannot always be recognized by bacteriologic studies alone. The surface of the skin and mucous membranes usually contains many types of microörganisms, some of which are definitely saprophytic while others are only facultatively pathogenic. Secondary infections of skin and mucous membrane lesions are of very frequent occurrence and often the inflammatory process producing the exudate is due to the action of more than a single type of microörganism. Therefore the laboratory should report only the various types of fungi or bacteria present and the final evaluation of their pathogenicity must depend upon clinical considerations and cytologic or histologic examination of the lesion in question.

COLLECTION OF MATERIAL

Certain general principles must be observed in obtaining and preparing material for examination. It is essential that the material be obtained

as fresh as possible. Crusts and superficial layer of purulent exudate should be removed by washing the lesion carefully with physiologic salt solution or even by applying a wet dressing for 24 hours. The exudate should be removed with a sterile wooden applicator, a cotton swab or a platinum wire loop. Material may also be obtained by scraping the surface of the lesions gently with a sterile scalpel or a small curette. Surface anesthesia with a 4 per cent sterile solution of cocaine may be necessary to permit the scraping. Small vesicles or pustules should be punctured with a sterile needle and the contents aspirated with a tuberculin syringe. It is important that any local treatment of the lesion be withheld for at least 24 hours before the material is obtained.

After the material has been removed it may either be examined directly under the microscope or be used for bacteriologic culture or animal inoculation. Under some circumstances moist smears should be prepared by adding a drop of physiologic saline solution, while for most purposes dry smears are sufficient. It is important that the material be spread thinly and evenly on the slide. Moist smears are recommended for examinations for fungi and trichomonas and for the darkfied examination of spirochetes.

Material from the eye should be obtained from the diseased parts only and any contact with healthy parts carefully avoided. Scrapings from corneal lesions and aspiration of the anterior chamber of the eye should be left to the skill of the specialist. Material from the ear should be collected only after the skin of the ear and the external auditory canal has been carefully cleaned in order to avoid contamination. Material from the nose may be obtained by having the patient blow the secretion into sterile gauze. Exudate from the various sinuses must be obtained by the trained specialist. Material from the oral cavity and pharynx can be obtained with sterile cotton applicators or a platinum wire loop. Sometimes it is advisable to probe gingival pockets or tonsillar crypts. Scabs and crusts must be removed from the tongue and gingiva and material taken from the underlying tissue surface.

Lesions of the genito-urinary tract are important sources of material for laboratory examinations and for their study we refer the student to the chapters on urine examination and the recognition of venereal lesions. Prostatic secretions can sometimes only be obtained after rectal massage of the prostate.

The examination of vaginal smears for the recognition of various lesions of the female genital tract has become increasingly popular and at the present time the examination of such smears constitutes one of

the important tasks of many laboratories. In addition to the presence of pathogenic microörganisms, the type of exudate and the condition of the exfoliated cells of the female genital tract are frequent subjects for examination. To obtain vaginal smears many different methods are available, each of which suits best one particular purpose. Aspiration of cells from the vaginal pool by means of a long pipette with a rubber bulb is perhaps the simplest method and often can be performed by the nurse or technical assistant. However, we have found it more satisfactory to always obtain vaginal smears under visual control and for this reason recommend a speculum and a strong light. If a viginal examination precedes taking of the smear, it is important that no lubricant be used either on the speculum or on the finger or gloves. *Ayres* has designed a specially shaped wooden blade which can be destroyed after the smear has been taken and which permits scraping the vaginal portion of the cervix and the endocervical os at the same time (Fig. 17.3). We recommend that two vaginal smears be taken from each patient, one to be obtained by scraping the exposed and well illuminated cervix with any type of scraping instrument, and one to be taken by pipette from the vaginal pool in the posterior vaginal fornix. Thus we obtain freshly exfoliated cells from the areas which are the most likely sites of pathology and at the same time also obtain cells which have exfoliated from other parts of the female genital tract, particularly the uterine fundus. Rapid fixation of the smear in the alcohol-ether mixture is essential if Papanicolaou stain is to be performed. When such cannot be done

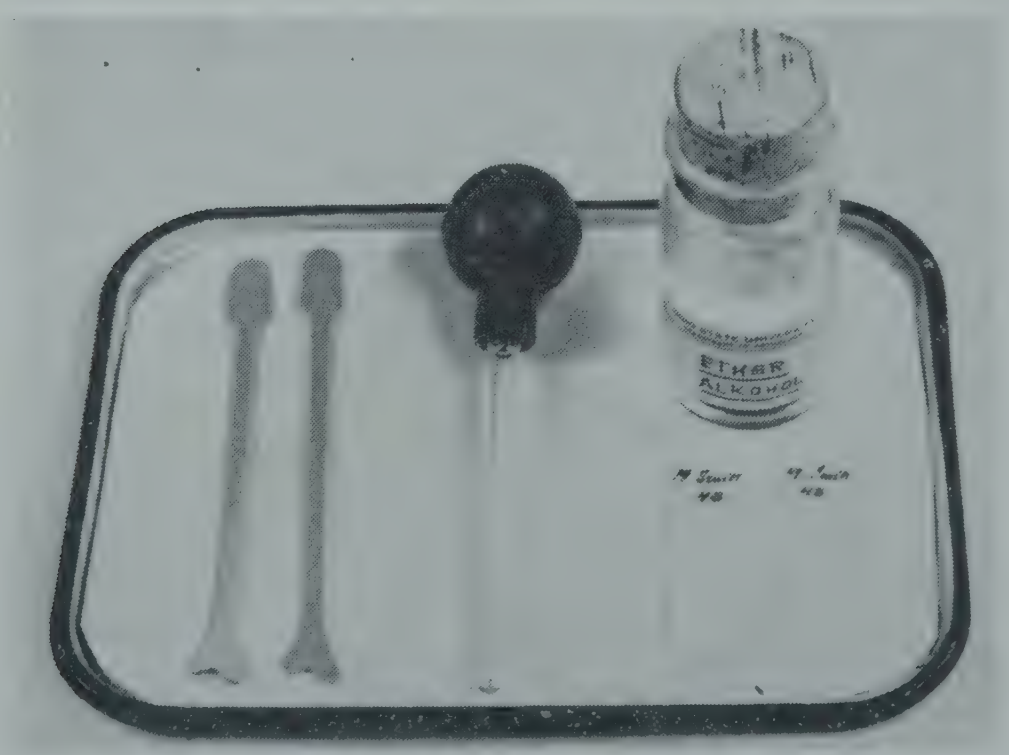


FIG. 17.3. Tray for the preparation of vaginal smears. Note cervical scrapers, vaginal pipette with suction bulb, glass slides and bottle with fixing fluid.

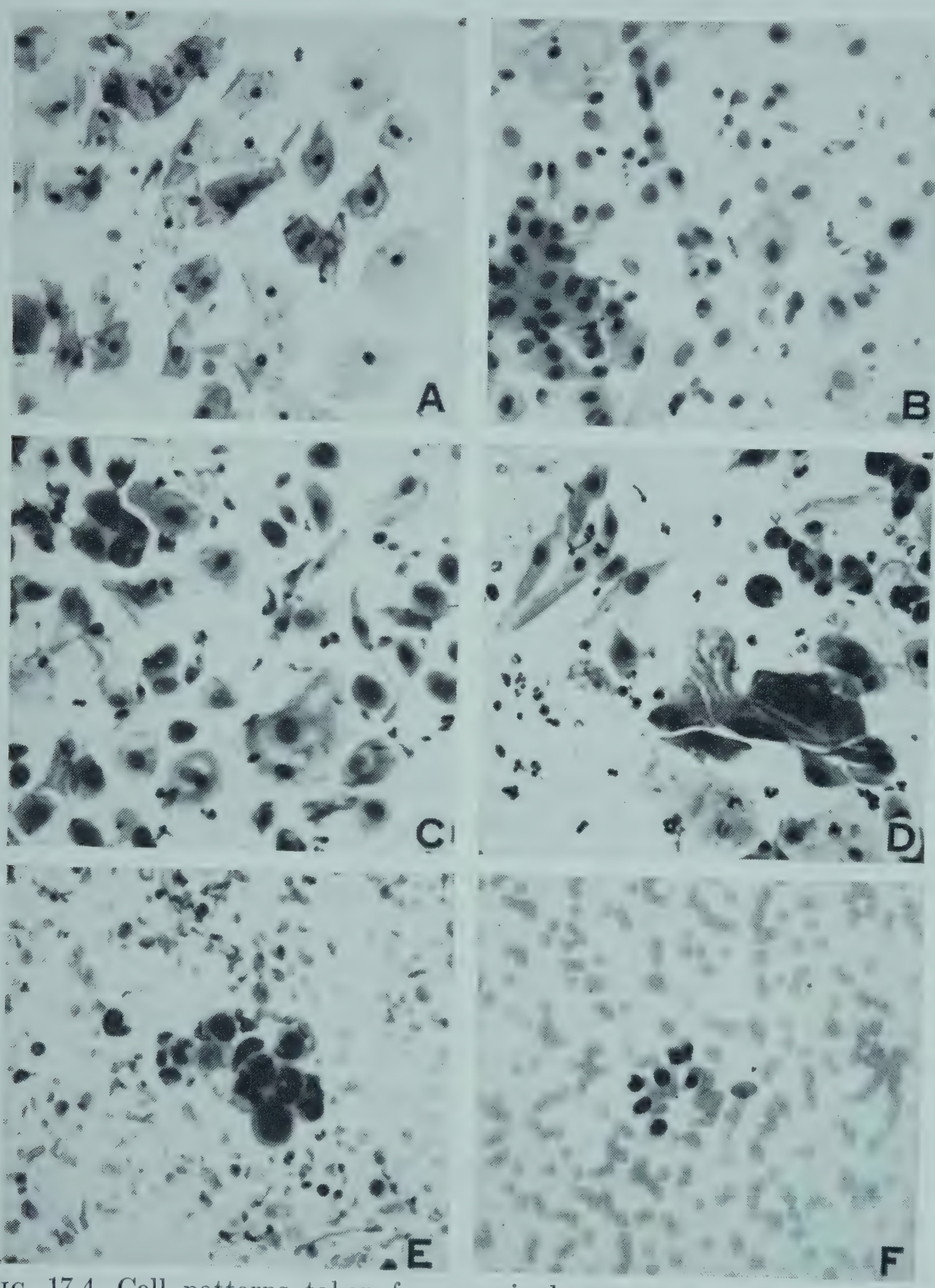


FIG. 17.4. Cell patterns taken from vaginal smears (Papanicolaou, ×200). A: Normal mature vaginal epithelium. B: Involutional vaginal epithelium. C: Atypical nonmalignant cells in cervical dysplasia. D. Malignant cells from well differentiated squamous cell carcinoma of the cervix. E: Malignant cells from undifferentiated squamous cell carcinoma of the cervix. F: Malignant cells from adenocarcinoma of the fundus.

immediately, the slide should either be left in the fixative or taken out and covered with a drop of glycerine, which will prevent dehydration.

In the evaluation of vaginal smears it is very important the the age of the woman and the time relationship to her last menstruation are recorded. Only if the pathologist knows these data will he be able to give a correct endocrinologic evaluation of the exfoliated cells.

Other regions of the genital tract of women which may be the site of pathologic processes are the Bartholin glands, Skene's glands and the female urethra. Here the interest usually centers around the presence of the pathogenic organisms of venereal diseases and the student is referred to Chapter 18 for details.

PHYSICAL CHARACTERISTCS

The quantity of exudates from the skin and mucous membranes is usually not excessive. The material is nearly always turbid and varies in color between white, gray, yellow, red and black. A deep green color of pus is suggestive of pyocyaneous infection and is often found in the middle ear. The odor is usually not particularly characteristic except in the presence of putrefaction. Because of the large amount of fibrinolytic enzymes exudates usually do not coagulate. Only in certain types of infection does coagulation take place producing membrane-like structures covering the infected areas. Chronic lesions with development of granulation tissue tend to bleed easily and form dark-colored crusts.

CYTOLOGIC EXAMINATION

The cells of exudates from skin and mucous membranes are best studied on thin smears fixed while wet in an equal mixture of alcohol and ether and stained by Papanicolaou's method. Staining of the smears with hematoxylin-eosin, Wright's or Giemsa's stain are less satisfactory. Red blood cells, although often present, are numerous only in granulating or deeply ulcerating lesions. Neutrophilic leukocytes represent usually the bulk of cells and are found diffusely scattered over the smear or clumped in small aggregates. They usually possess mature lobated nuclei and their cytoplasm may show phagocytosis or toxic changes. Eosinophilic leukocytes are found frequently in nasal secretions and indicate an allergic background for the exudate. Lymphocytes are present in material from chronic granulomatous lesions. Endothelial cells are found in varying concentrations in all types of lesions and often show phagocytosis and degenerative changes. Epithelial cells

from the skin and mucous membranes are always present in their characteristic types and often show vacuoles and other degenerative changes. Smears from the skin and mucous membranes lined with squamous cell epithelium show exfoliated epithelial cells in varying stages of keratinization as evidenced by the size of the nuclei and the staining reaction of the cytoplasm. This is especially important for smears from the vagina and the buccal mucosa, where important pathological conditions can be recognized by deviations from the normal process of keratinization (Papanicolaou, Montgomery).

Necrotic amorphous material, particles of keratin, hair-shafts and a network of fibrin form usually a rather non-specific background for the cells together with a mixed flora of all types of bacillary and coccal organisms.

Smears from superficial neoplasms involving the skin and mucous membranes will contain malignant cells, the recognition of which has become an important tool in the diagnosis of cancer.

With regard to the technic employed in the study of exfoliative cytology we wish to emphasize that the preparation of the smear is at least of equal importance as the staining and study of it. The smears should be taken preferably from scrapings of the suspected lesion or material aspirated from its immediate surroundings. They must be fixed in moist condition in the alcohol-ether mixture and should be neither too thick nor too thin. Malignant cells can be recognized by the characteristics discussed in the preceding chapter. Nuclear atypism is not an exclusive property of malignant cells, as Ferguson has pointed out. However, it is probably most commonly associated with malignant growth and therefore has the greatest diagnostic value.

In an attempt to standardize examination of exfoliated cells Papanicolaou has suggested five different classifications: Grade I, representing smears with normal cells only; Grade II, referring to slides showing evidence of inflammation and benign hyperplasia. Grade III is used for those slides in which some atypical cells appear making the examiner "suspicious" of the presence of a malignant lesion. In slides labeled Grade IV the evidence of atypical malignant cells is established, while in smears classified as Grade V these malignant cells are overwhelming in number. In our own laboratory we avoid as much as possible a diagnosis of "cells suspicious of malignant change" and try to reach a decision between positive or negative diagnosis by repeating the smears until we feel that a definite diagnosis can be made.

The past year has seen a wide application of this method to the diag-

nosis of cancer. The recognition of malignant tumors of the oral cavity (Montgomery and von Haam), the upper respiratory tract (Woolner and McDonald), and the cervix and uterus (Papanicolaou) by the study of exfoliative cytology has been firmly established by numerous laboratories. The diagnostic reliability of this method with regard to tumors in these three regions is now considered nearly equal to the accuracy of biopsy diagnosis. Malignant tumors of the stomach, the rectum, and the genito-urinary tract also have been successfully diagnosed by the recognition of malignant cells in the gastric washings, rectal smears, and catheterized urine, but the results are at present too varying to recommend it as a diagnostic method.

Follow-up studies of patients with carcinoma of the cervix have shown that the exfoliated epithelial cells reflect faithfully the local changes of the malignant lesions under radiation therapy. Local recurrence of the tumor can also be recognized early and accurately by this method and the evaluation of the inflammatory reaction, the hemorrhage and the bacterial flora permits certain guarded prognostic conclusion with regard to the progress of the neoplastic process.

The study of the exfoliative cytology of the vagina by frequent vaginal smears has also been recommended as a routine procedure for the early recognition of cancer of the cervix (Reagan). The screening out of obviously normal smears by specially trained laboratory technicians has been advocated by some and opposed by others. Boyd rightly cautions against the false security such a test may give if performed by negligent or unskilled personnel. In the hands of the careful and experienced examiner, however, the study of the exfoliative cytology of certain regions—particularly the vagina and oral cavity—has a definitely established value in the recognition and follow-up of mucosal surface pathology (von Haam).

BACTERIOLOGIC EXAMINATION

Bacteriologic examination of exudates from the skin and mucous membranes represents one of the more important tests of clinical pathology. Here as in perhaps no other field the clinical pathologist can solve puzzling diagnostic problems and give pertinent advice with regard to the prognosis and treatment of the lesion. Two conditions must be fulfilled to make such bacteriologic studies successful. The material must have been collected in correct fashion with the avoidance of all contamination, and the clinical pathologist must have a thorough knowledge of all methods of diagnostic bacteriology. The first condition has been

discussed in a preceding paragraph of this chapter. For the second we refer the student to the chapter on Medical Bacteriology.

HISTOPATHOLOGIC EXAMINATION

The chapter on the clinico-pathologic examinations of skin and mucous membrane lesions would not be complete without briefly discussing the indication and value of the biopsy as a diagnostic method. Although its use has not been as popular as the bacteriological and cytological methods of laboratory examination, it is being more widely used in recent years and is now recognized as a definitely established method of laboratory diagnosis. The two main indications for biopsy of a lesion of the skin or mucous membrane are the impossibility of reaching a satisfactory diagnosis by any other method, and the suspicion of a malignant process. Conditions for the successful biopsy must be the proper selection of the case and a faultless technic. Small lesions may be excised but should not be biopsied. In large lesions the biopsy should include the margin of the lesion and should be deep enough to include healthy underlying tissue. The biopsy must be performed under local anesthesia and careful observance of strict asepsis. It is a surgical procedure and requires surgical training for the operator and surgical postoperative care for the patient. Contraindications for biopsy are the general condition of the patient or an acute, very virulent pyogenic infection of the lesion. In contrast to some authors' opinion, it should be emphasized here that the suspected presence of a malignant process is an indication rather than a contra-indication for biopsy. The danger of spreading the malignant process is minimized if the surrounding tissue is blocked off by infiltrating anesthesia. For additional precaution removal of the tissue by the electric knife may be recommended. For the early diagnosis of malignancies, which is the absolute requisite for a hope of cure, the biopsy is at present the most reliable method of examination and its frequent use cannot be recommended too strongly (von Haam).

CLINICO-PATHOLOGIC CORRELATION

Skin. In the skin the variety of exudative, ulcerative and indurative lesions often presents immense diagnostic difficulties and assistance furnished by laboratory methods leaves much to be desired. Biopsy of the skin lesion is becoming increasingly regarded as the most reliable method available for diagnosis. However, the biopsy alone cannot do justice to the numerous different skin diseases which are only known to

the dermatologist by their weird names and which are often indistinguishable from each other by the microscope. Any student of the numerous recent textbooks on histopathology of the skin will agree that comparatively few skin lesions with the exception of the neoplasms are characterized by an outstanding histopathologic picture. In the attempt to recognize etiologic factors of skin diseases one is confronted by the maze of saprophytic bacteria and fungi which inhabit the normal skin and which make the selection of the proper pathogenic organism difficult if not impossible. Fungus diseases of the skin play a very important role and the recognition of the pathogenic fungus has often been the task of the laboratory. Here cultural methods must usually be employed although in very typical cases examination of dry smears or moist preparations may produce the desired diagnosis. For the recognition of fungus diseases we refer the student to Chapter 13 on Medical Mycology. Neoplasms of the skin are now recognized readily by biopsy. It is important the the biopsy be taken deep enough to permit not only the study of the epithelium but also the underlying dermal structures. Small skin lesions should not be biopsied but excised in toto. Specific infectious diseases which can be recognized by a skin biopsy or skin culture are: leprosy, tuberculosis, syphilis and some of the virus diseases which produce characteristic inclusion bodies, such as herpes simplex and molluscum contagiosum. In many instances, while not actually pointing to the diagnosis, the biopsy of the skin lesion will make it possible to exclude many diseases, which in itself is an important aid to the diagnostician. Among the large group of noninflammatory dermatoses only psoriasis, lupus erythematosus and lichens planus can be recognized with some certainty by biopsy, while in the many other types of so-called chronic dermatitis, or eczema, the histologic picture is at best only suggestive (Lever).

Eye. Purulent material obtained from the conjunctiva or cornea usually contains many neutrophilic leukocytes and epithelial cells. Eosinophils may be found abundantly in exudates of allergic conjunctivitis. The presence of gonococci would definitely indicate gonococcal infection of the eye. The Koch-Week's bacillus is the cause of the acute epidemic conjunctivitis known as "pink-eye" and is found in large numbers both extra- and intracellularly in smears of the diseased conjunctiva. The Morax-Axenfeld bacillus (*Moraxella lacunata*) is found in short chains in the exudate from subacute or chronic forms of conjunctivitis particular in the area of the caruncle and the outer angle of the eye. Diphtheria bacilli may produce a pseudomembranous conjunctivitis

though at times such conjunctivitis may also be produced by streptococci.

Ear. Purulent material obtained immediately after perforation of the ear drum is of considerable value in determining the specific cause of otitis media. The bacteria usually found are pneumococci, streptococci, staphylococci, Friedlander's bacilli, *Bacillus pyocyaneus*, and *Escherichia coli*. Smears made later from the discharge of chronic otitis media or chronic mastoiditis usually show contaminating bacteria and are of little value.

Nose. Smears made from purulent material obtained from the maxillary, frontal or ethmoid sinuses may be of value in determining the cause of chronic purulent type of sinusitis. A very satisfactory method for the diagnosis of leprosy is the finding of characteristic acid-fast lepra bacilli (*Mycobacterium leprae*) in smears or scrapings from lesions on the turbinate bones. Smears from the nose stained with Wright's stain show characteristic eosinophilia in season allergic conditions such as hay fever, while in the perennial type of nasal allergy eosinophils are seldom found.

Mouth and Throat. Depending upon the cleanliness of the mouth, various numbers and types of bacteria are found in the normal oral cavity, including staphylococci, streptococci, pneumococci, fusiform bacilli, various spirochetes and leptothrix or monilia. The finding of Vincent's organisms in smears of the gingiva or the buccal mucosa should only be considered significant in the presence of pathologic lesions. Thrush, which is caused by *Endomyces albicans* and which occurs most frequently in infants, is characterized by white furry patches on the mucous membrane of the tongue, gums and cheeks. The fungus appears as a network of single or branching mycelian filaments with budding cells similar to yeast cells. Malignant lesions of the oral cavity can be diagnosed by examining smears prepared from scrapings of the lesions. However, considerable experience is required for this type of diagnosis and a biopsy offers an easier and in most instances a more accurate diagnosis.

Respiratory Tract. Examination of the sputum is important for the diagnosis of all infectious diseases of the respiratory tract with the exception of the virus diseases (Chap. 22). Bronchial asthma, bronchiectasis, pulmonary mycosis and chronic passive congestion of the lung can be also well recognized by the characteristic sputa with the appearance of eosinophils, Charcot-Leyden crystals, fungus colonies and heart failure cells. Malignant tumors of the respiratory tract can be recognized by the appearance of malignant cells in the sputum or the bronchial aspira-

tions. This method in combination with other clinical data and x-ray findings is yielding better results than the often futile and painful attempts to procure a biopsy through the bronchoscope. For this purpose we prefer material secured through the bronchoscope to simple sputum. We can recommend the examination of smears stained by Papanicolaou's method as well as slides prepared from tissue blocks imbedded in paraffin The use of gelfoam sponges to trap malignant cells has not proven successful with us.

Gastrointestinal Tract. Atrophic gastritis and gastric achylia can be recognized in smears prepared from gastric washings by the appearance of intact desquamated squamous cells from the mouth or esophagus, lymphocytes and mucus-secreting gastric cells. Pernicious anemia can be diagnosed by the demonstration of large pale cells of the gastric mucosa (Rubin), while in carcinoma of the stomach one may or may not find groups of atypical cells characterized by marked dyskariosis. Smears secured from rectal lesions with the proctoscope will show inflammatory cells in the presence of ulcerative processes of the colon. Vegatative forms of *Endameba histolytica* may be recognized in wet smears prepared from ulcerative lesions of the rectum and sigmoid when examined immediately and preferably on the heated microscope stage. Carcinoma of the rectum and sigmoid may yield clusters of atypical cells but this method is far too uncertain to replace the biopsy in diagnostic importance.

Genito-Urinary Tract. Inflammatory processes involving the kidneys, bladder and male sex organs can well be recognized by examination of smears prepared from catheterized urine, material obtained after prostatic massage or smears from urethral lesions. Colon bacilli, streptococci and staphylococci are the most common organisms present in pyelonephritis and cystitis. Urinary tuberculosis can best be diagnosed by inoculating urinary sediment in guinea pigs. Acute and chronic prostatitis will show the usual pyogenic cocci and pus cells in smears obtained after prostatic massage. The gonococcus is the most common cause of acute inflammatory conditions of the urethra. When gonococci are not found on repeated examination of urethral secretions, in spite of the presence of pus cells, the infection is usually referred to as nonspecific urethritis.

Malignant tumors of the kidney, bladder and prostate may be recognized by the presence of atypical cells in smears prepared from the sediment of catheterized urine, prostatic secretion or material obtained by ureteral catheterization. At present we consider this method too uncertain to be of great diagnostic value.

Female Genital Organs. Infectious processes of the female genital organs are usually caused by gonococci, chancroid bacilli or pyogenic microörganisms. The differential diagnosis of these lesions is discussed in Chapter 18. *Trichomonas vaginalis* also can be the cause of inflammatory conditions of the vagina and female urethra. Vaginal smears are equally as useful as an endometrial biopsy for study of the endocrine status of the female genitalia. Lack of estrogenic or luteinizing hormones or their overabundance can be demonstrated by the type of desquamated vaginal epithelial cells, and vaginal smears properly correlated with the menstrual cycle and the age of the woman will be of great value in the recognition of female endocrinopathies. Juvenile hypogonadism, menopausal states, therapeutic or pathologic hyperestrinism are some of the conditions which may be recognized by study of vaginal smears. Another important field is the early diagnosis of malignant processes of the cervix and the uterus, which may be recognized by the vaginal smear method in the precancerous or noninvasive stages. The accuracy of this method varies of course with the experience and the exactness of the individual examiner, but false negative results should not be obtained in more than 10 per cent and false positive results in not more than 1 per cent of the cases examined, which represents a far greater accuracy than can be obtained by any other method of examination except that of repeated biopsy. The vaginal smear also lends itself well to follow-up studies of radiation therapy of cervical carcinoma and to the early recognition of recurrent malignancy following surgery. The value of the examination of vaginal smears in gynecologic practice can be considered as definitely established and recent years have witnessed its wide acceptance as a valuable diagnostic method.

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CHAPTER 18

THE DIAGNOSIS OF VENEREAL LESIONS

By Emmerich von Haam

GENERAL CONSIDERATIONS

Clinical methods for the diagnosis of venereal diseases are admittedly unreliable, and only in the most typical case is the lesion sufficiently characteristic for a correct diagnosis. The clinical laboratory provides the most valuable methods for the diagnosis of venereal lesions, chiefly by demonstrating the causative organism. This can be accomplished by the various diagnostic bacteriological methods including study of smears from the lesion, cultural methods, and animal inoculation while systemic reactions can be studied by immunological and histopathological methods (Table 18.1).

SYPHILIS

Clinical Correlation. The laboratory tests used in the diagnosis and treatment of syphilis depend upon the stage or localization of the disease in a presumed syphilitic infection. By means of the laboratory, the presence of syphilis can be shown by finding the *Treponema pallidum* in the discharge, blood, spinal fluid or tissue of infected patients, by recognition of the specific local inflammatory process through biopsy, and by recognition of the specific immune biological process by serological methods.

Treponemata pallida are present in enormous numbers in the early lesions and their demonstration is the most important diagnostic test in this stage of the disease. Beginning with the third week, a general antibody reaction causes a retardation in the rate of spirochetal multiplication with a consequent decrease in numbers to the point where it may be impossible to demonstrate them.

In generalized syphilis or in the stage of widespread invasion of the tissues by the *Treponema pallidum*, the complement fixation or precipitation tests are the most valuable tests in diagnosis. In neurologic syphilis the tests of value include the complement fixation test, the precipitation test, cell count, globulin determination, and colloidal gold reactions on spinal fluid. For detailed consideration of these see Chapters 19 and 20.

TABLE 18.1

The Diagnostic Value of Laboratory Methods for the Diagnosis of Venereal Disease

Venereal disease	Stage	Demonstration of the Infectious Agent in:					Demonstration of Host Reaction by:				Clinical Appearance of Lesions
		Dark-field	Stained smear	Tissue	Culture	Animal inoculation	Complement fixation	Precip. and floc. tests	Allergic skin reaction	Specific histo-path.	
Syphilis	Primary	+	+	+	−	?	?	+	−	?	Not typical
	Secondary	+	+	+	−	?	+	+	−	+	Typical
	Tertiary	−	−	?	−	−	+	+	−	+	Not typical
Gonorrhea	Acute	−	+	−	+	−	?	−	−	−	Not typical
	Chronic	−	?	−	+	−	+	−	−	−	Not typical
Chancroid	Acute	−	+	−	+	−	−	−	+	−	Not typical
	Chronic	−	?	−	+	−	−	−	+	−	Not typical
Venereal fusospirochetosis	Acute	+	+	+	−	−	−	−	−	+	Not characteristic
	Chronic	+	+	+	−	−	−	−	−	+	Not characteristic
Granuloma inguinale	Acute	−	+	+	?	?	−	−	−	?	Typical
	Chronic	−	?	+	?	?	−	−	−	?	Typical
Lymphopathia venereum	Acute	−	−	−	−	+	+	−	+	+	Not typical
	Chronic	−	−	−	−	+	+	−	+	+	Not typical

+ = valuable for diagnosis; ? = of questionable value; − = of no value.

Obtaining of Material. The worker should take all precautions against infection by wearing rubber gloves, and infectious material should be placed in a disinfecting solution immediately after use. The patient is usually placed in a reclining position with complete exposure of the lesion. The skin surface on and about the lesion should be thoroughly cleaned with sterile gauze moistened with water. If this can not be done, it is best to instruct the patient to return next day after soaking his lesions repeatedly in a hot weak salt solution. If the lesion is covered with a dry crust, it is necessary to remove the crust and if it is dry, it is necessary to scrape the surface with a scalpel until a slight epithelial defect is produced through which serous exudate oozes on pressure. The specimen should always be taken from the deeper part of the lesion, and many failures are attributed to superficial careless scraping of the ulcer. To avoid dilution with blood the lesion should be squeezed between the fingers of the left hand (Fig. 18.1). This also serves to lessen the pain incidental to scraping. If necessary, application of a pledget of cotton moistened with 2 per cent novocaine solution for several minutes results in a fairly good anesthesia.

The scraped material on the knife blade is placed directly on a thin microscope slide and the cover glass applied. The scrapings should be examined at once. It is not advisable to dilute this material for examination.

The serous discharge from intra-urethral lesions may be examined without scraping. A dorsal slit can be made if the foreskin of the penis is swollen and indurated to such an extent that the lesion can not be exposed. Mucous patches within the mouth may be examined if

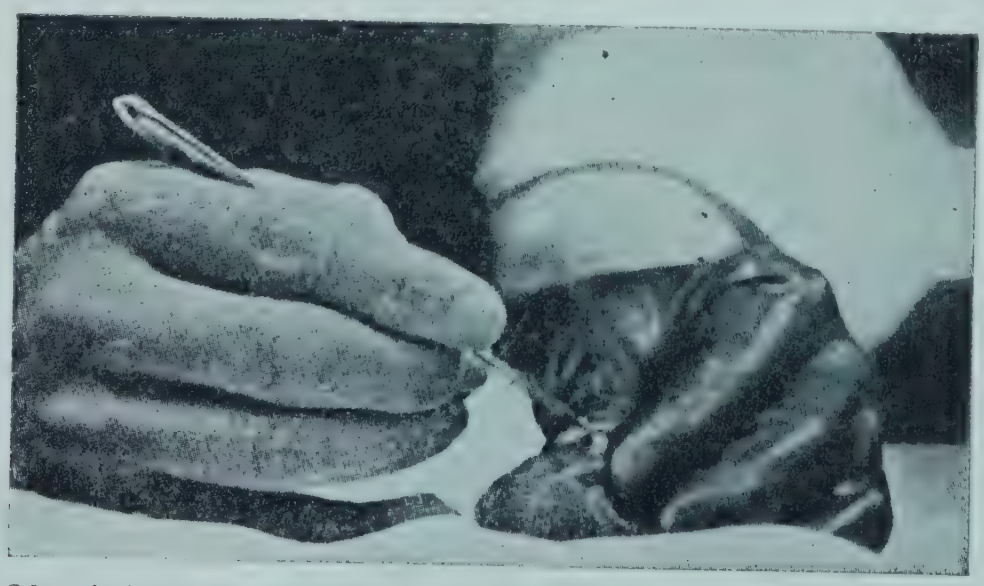


FIG. 18.1. Obtaining material for dark field examination. Sufficient pressure is maintained with the gloved hand at the base of the lesion to prevent bleeding, and squeeze out the clear fluid which contains large numbers of *Trepomena pallidum.*

surface contamination with mouth spirochetes can be avoided. Condylomata about the vulva and rectum are usually fissured and contaminated with nonpathogenic spirochetes. Chancres rarely develop within the vagina. Mucous patches on the cervix may be exposed with the vaginal speculum and the profuse serous discharge may easily be obtained with a long tip capillary pipette and rubber bulb for suction.

Enlarged lymph glands adjacent to primary lesions may be aspirated with a large (18 gauge) needle attached to a dry glass syringe after sterilization of the skin with tincture of iodine and alcohol. The gland is held between the fingers of the left hand and the needle inserted. There are always fewer spirochetes in this material and the search for them should be prolonged.

Demonstration of Treponema pallidum

Dark Field Examination. Principles. This test was introduced in 1906. The principle consists of a strong lateral illumination of refractile particles against a dark background. This is accomplished by a dark field, or "paraboloid" condenser, an illuminating device replacing the "Abbé" condenser of the microscope, whereby a powerful cone-shaped beam of light can be focused on the small drop of fluid mounted on a slide under a cover glass (Figs. 18.2 and 18.3). Since these light rays are projected through the preparation at an angle of 45° from the axis of the microscope, they are not visible except when refracted upward by some translucent structure in the preparation such as a leukocyte or a bacterium suspended in the fluid. The "burr" of light thus refracted from small particles is visible as a shining, refractile object. Because ordinarily invisible objects are thus made visible, the dark field is also known as the "ultramicroscope."

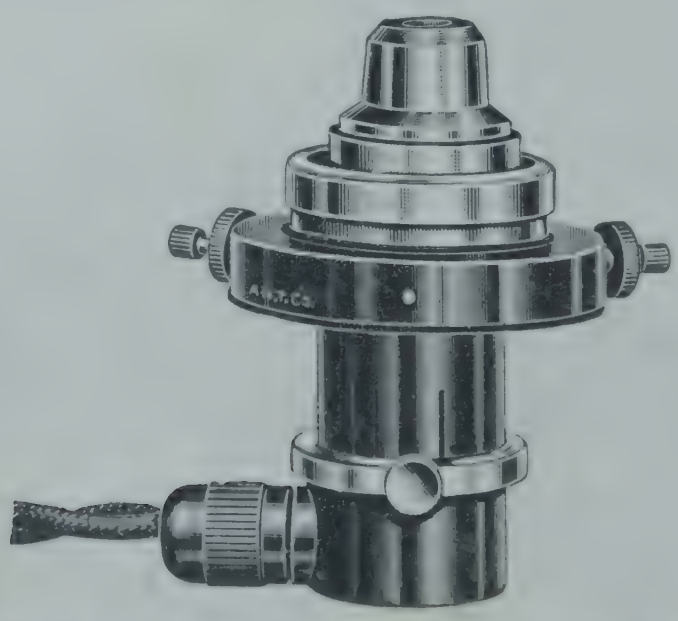


FIG. 18.2. A satisfactory type of dark field equipment which fits into the substage of the microscope. The light is in the lower part.

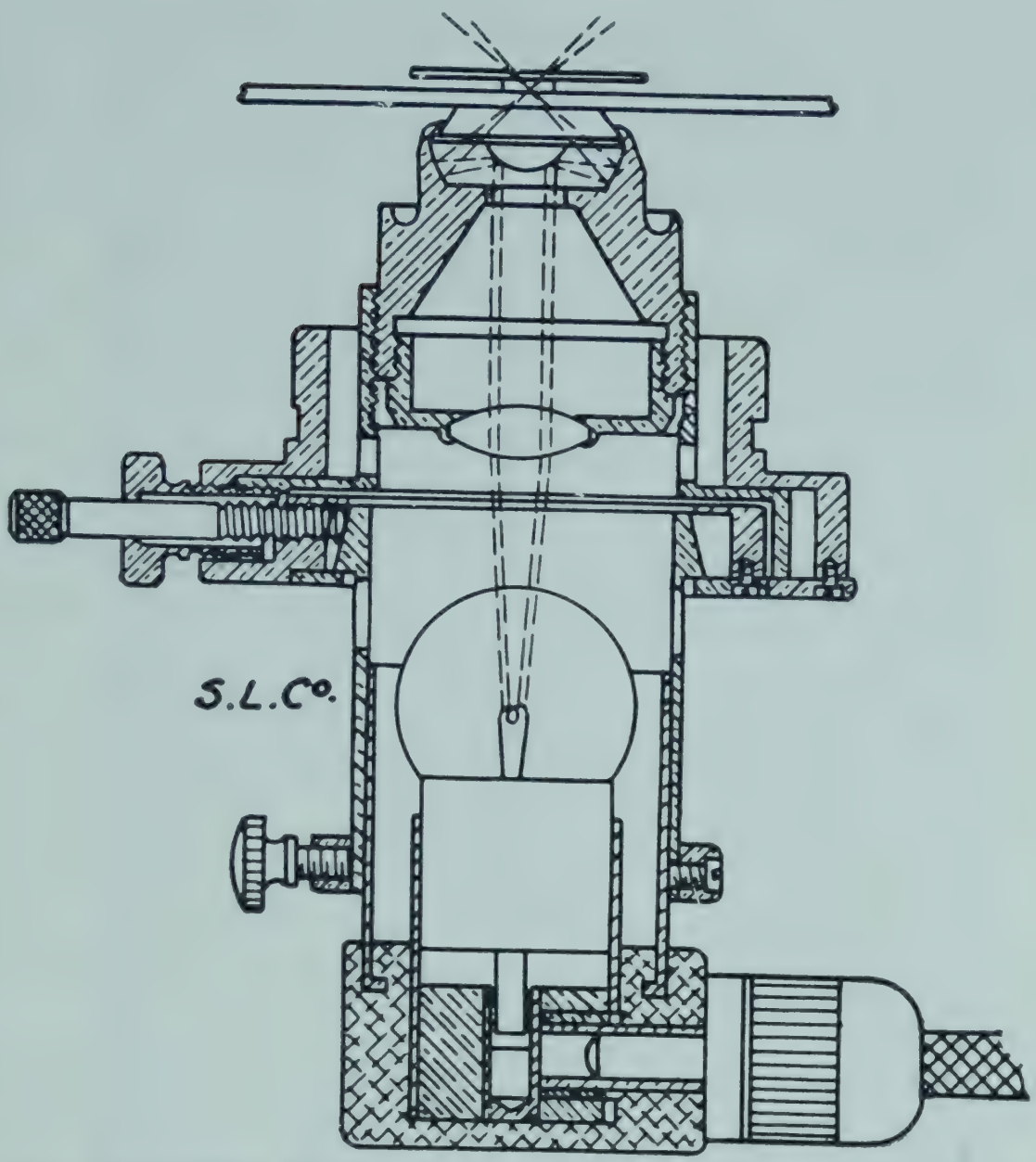


FIG. 18.3. Illustrating the path of light rays through a dark-field condenser

Recognition of the Treponema pallidum. The *Treponema pallidum* is about twice as long as the diameter of a red cell, the average length being about 15 micra. The curves are small, regularly spaced, and number from ten to fifteen in the average organism (Fig. 18.4). When seen in its motile state in the dark field, it appears as a slowly revolving coil spring and in addition it may move forward, backward or laterally, the motion being relatively slow and in any direction. There seems to be a fixation or rigidity of the organism so that it does not bend nor assume an undulating motion. The queen of spirochetes has appropriately only a stately and unhurried travel and is never fitful or spasmodic in its motion (Cares). Aside from its mode of locomotion the *Treponema pallidum* has an almost unlimited scope in changing its outline. The changes in form can be classified as flexion, buckling, fluctuation, compression and expansion of its coils and looping (Cares). The number of spirochetes present varies with the age and type of the lesion. In early acute cases five or more spirochetes can be found in every field, while in older lesions much diligent search may be required to find a

single organism and this is especially true when local treatment preceded the examination.

Dyes. Smears can be stained with Wright stain. The stain should be permitted to act upon the smear for 30 minutes in order to bring out the delicate structure of the spirochetes. Goldsworthy and Ward recommend two simple and reliable methods for the staining of spirochetes using Grübler's Victoria blue 4R and carbolgentian violet. They claim a one hundred per cent agreement with results obtained by dark field examination and are enthusiastic about the great convenience of their use.

India Ink. The India ink method is useful in laboratories not equipped with the dark field instrument, but its faulty application has probably been responsible for many erroneous results. Although recommended in many textbooks as a simple and reliable method, it requires more skill for its application than is usually indicated. The common types of India ink on the market are too coarsely granular and too often contaminated with fungi or vegetable fibers to be useful and the same may occur in good grades of imported ink if it is too old. A smooth, even, brown background is absolutely necessary to bring out the white contours of the Treponema.

Knisely recommends the use of a colloidal aqueous solution of silver known under the trade name Collargolum. Slides are prepared in the same way as with the India ink method and the dried smear examined under the microscope. The unstained *Treponema pallidum* stands out distinctly against a background of dark yellow to light brown. Slides so prepared represent permanent records.

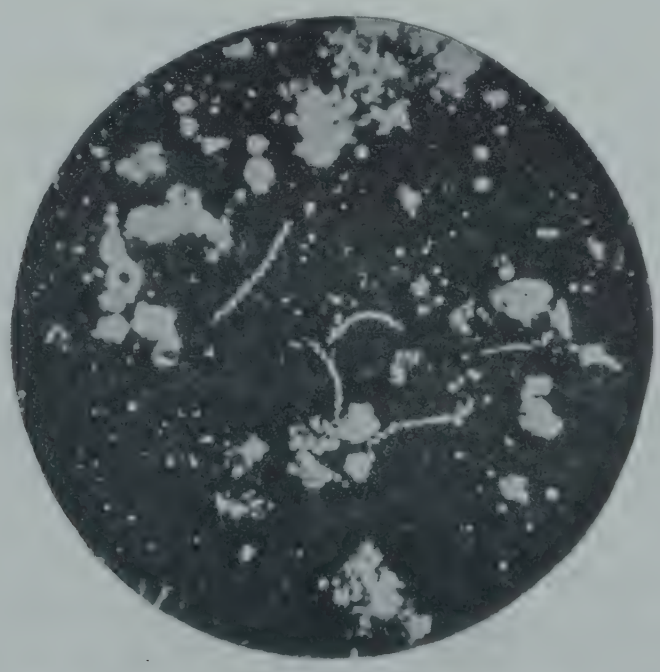


FIG. 18.4. Photomicrograph showing the *Treponema pallidum* in the dark field

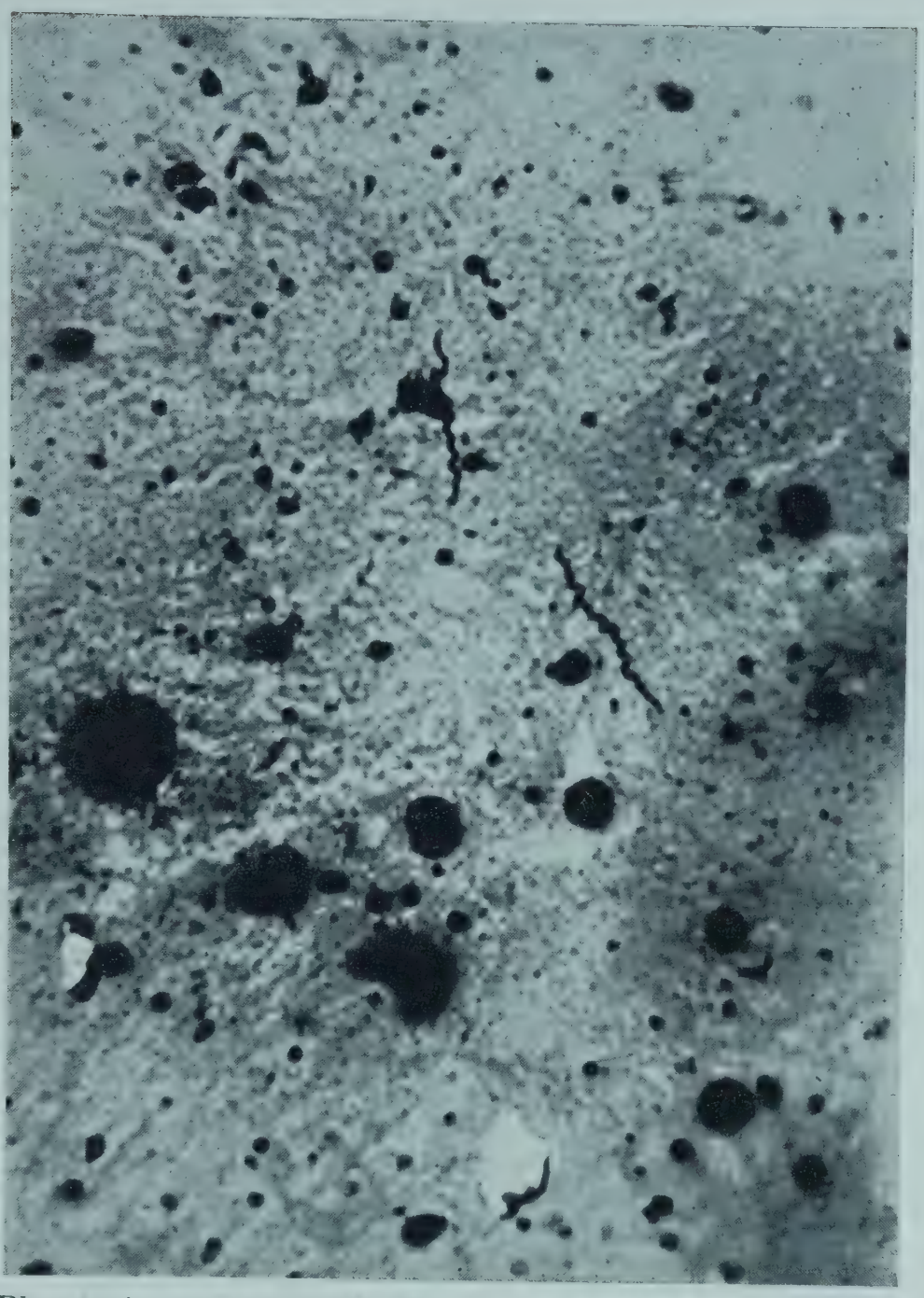


FIG. 18.5. Photomicrograph of the *Treponema pallidum* stained with Fontana's method in the smear from a chancre.

Fontana Stain. Undoubtedly the best result is obtained in a dry smear by impregnating the organisms with various silver preparations and the most reliable one is the method of Fontana. It stains spirochetes deeply black and permits a permanent record of the diagnosis (Fig. 18.5).

Demonstration of Spirochetes in Tissues. The number of spirochetes in syphilitic tissue, especially in congenital syphilis, is often astounding. In the lesions of acquired syphilis, they have been demonstrated in large numbers in the primary and secondary lesions (Fig. 18.6), but in the tertiary forms and in neurosyphilis their demonstration is difficult and sometimes impossible. The best methods for the silver impregnation of the Treponema in tissue sections are those of Levaditi and of Warthin and Starry. Levaditi's method is more reliable although it can not be

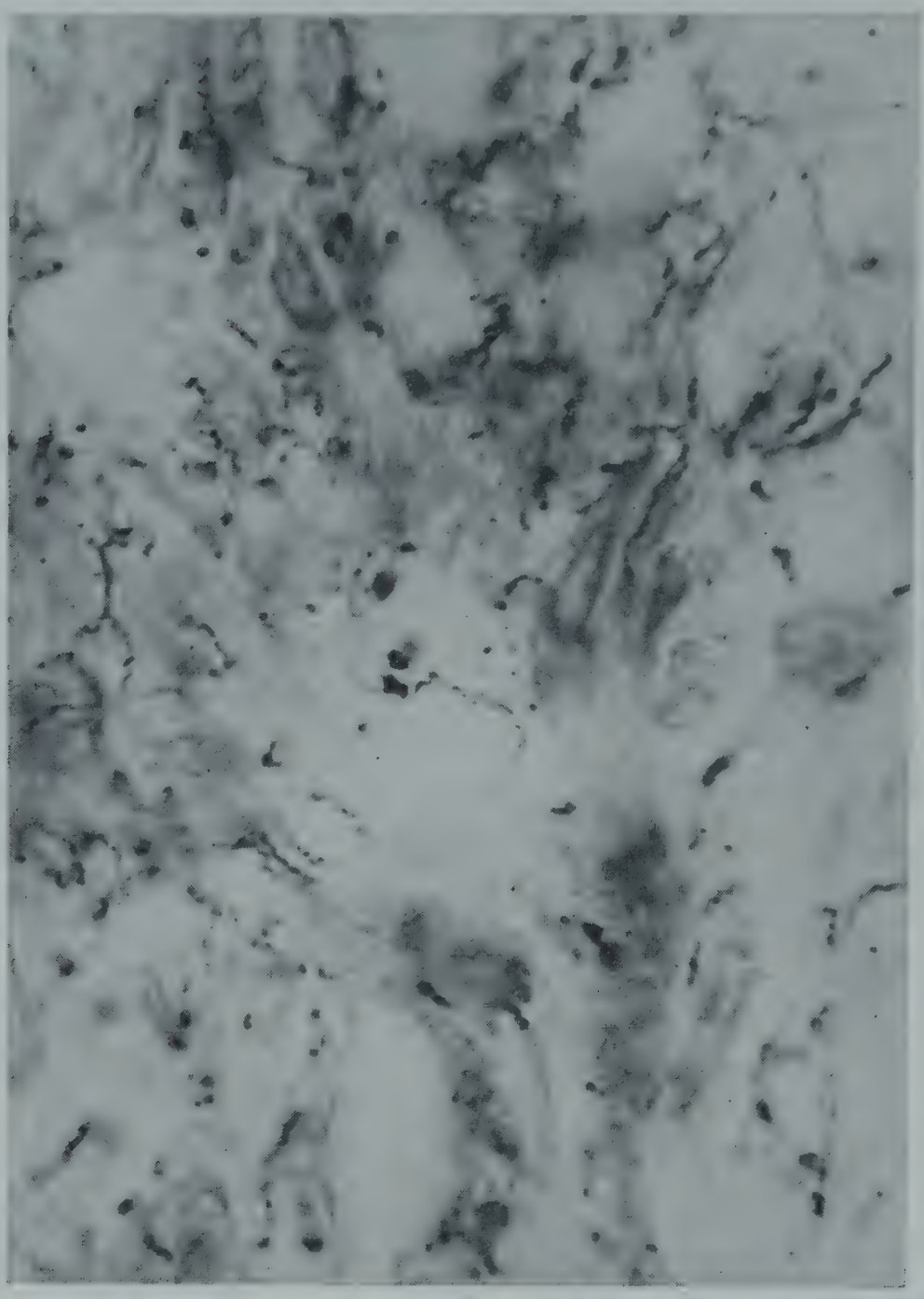


FIG. 18.6. Photomicrograph of *Treponema pallidum* visualized in the tissue by Levaditi's method.

used on tissues that have been imbedded in paraffin. Steiner's silver mirror method is excellent for frozen sections of formalin-fixed tissues and can be performed in about one hour. For details of these methods the reader is referred to books on histological technic.

Demonstration of Treponema pallidum by Animal Inoculation. Material suspected of harboring spirochetes may be inoculated into the testicles of adult rabbits. Three to four weeks later a syphilitic orchitis will appear which can be recognized by indurated swelling of the testicles. Smears prepared from the tissue show innumerable typical and very active spirochetes in the dark field. Because of the time required for the development of the infection in the rabbit its practical value as a diagnostic method is very limited.

Evaluation of Serological Tests for the Diagnosis of Syphilitic Lesions

The technics and individual merits of the complement fixation and the flocculation reaction in their numerous modifications are discussed in detail in Chapter 19 of this textbook. It is now considered generally accepted that for a correct serodiagnosis of syphilis two approved tests should be performed on each specimen and that the choice of the tests is less important than their execution. For this reason frequent checks on the accuracy of the tests as performed in the various laboratories is necessary and is being advocated by public health officials all over the United States. Properly conducted, all approved serological tests for syphilis possess a degree of specificity between 99 and 100 per cent while their sensitivity depends largely upon the stage of the infection. Primary chancres up to one week's duration will rarely give a positive serological test, while in lesions of secondary syphilis the positive serological tests will be 100 per cent. In tertiary syphilis the sensitivity will be between 85 and 90 per cent. Biological false positive reactions have been observed in yaws, leprosy, malaria, other spirochetal diseases and certain febrile states. False negative reactions are of course observed in all stages of syphilis and must not be permitted to override clinical judgment (Kolmer). Spinal fluid examinations have more diagnostic value in the late stages of syphilis and should always be included in the immune biological survey of the syphilitic patient. (See Chapter 20.)

The Histopathology of Syphilis

Although seldom employed as a diagnostic procedure, the histopathological change in the syphilitic lesion is a valuable addition to diagnostic methods especially in cases of tertiary luetic lesions in which the dark field examination usually, and the serological tests often, may give negative results. The "syphilitic reaction" seen in the primary chancre is mainly an endarteritis and periarteritis of the smaller blood vessels with narrowing of the lumen, marked proliferation of capillaries, and an infiltration of the perivascular lymph spaces with mononuclear cells, endothelial cells, lymphocytes, and plasma cells around the affected vessels. In the secondary forms of syphilis this reaction may be diffuse in the submucous layer of the external genitalia or in the form of small nodular accumulations of cells which often contain typical Langhans giant cells (Fig. 18.7). In the tertiary period of the disease vascular damage is usually more pronounced and more localized. Obstruction

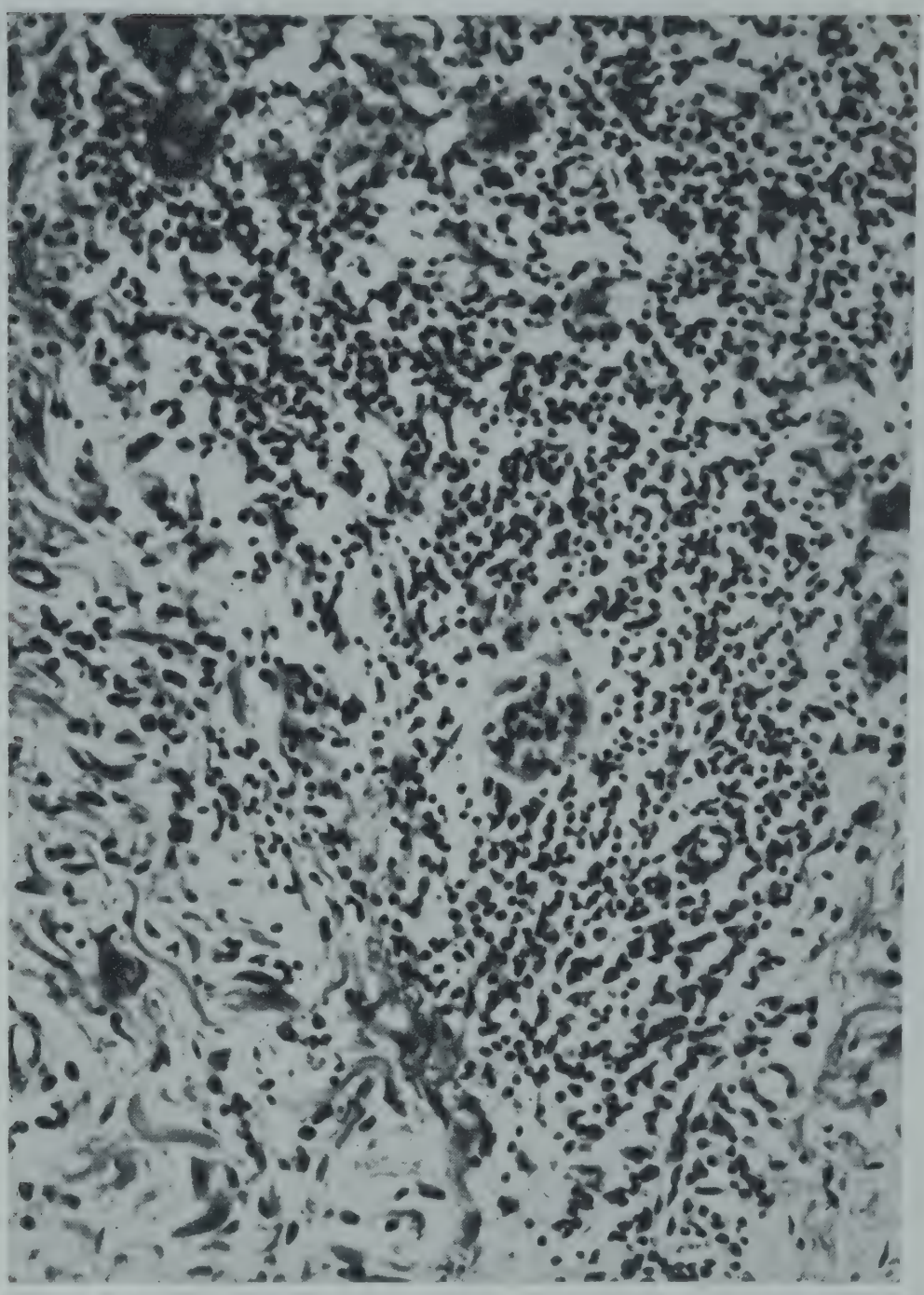


FIG. 18.7. Photomicrograph showing the typical histopathology of a syphilitic lesion.

of the blood supply caused by narrowing of the blood vessels is followed by circumscribed necrosis of the affected tissue with the development of a very vascular granulation tissue around the areas of necrosis. The marked predominance of plasma cells, the typical changes in the smaller vessels, and the vascular granulation tissue surrounding the necrotic zone of the gumma are the principal histopathological signs of the disease.

GONORRHEA

Clinical Correlation. The gonococcus is the most common cause of inflammatory states of the genito-urinary tract. In the male it is known to cause urethritis, prostatitis, vesiculitis, epididymitis, and orchitis. In the female it causes urethritis, bartholinitis, cervicitis, salpingitis,

and oophoritis, and in the infant female it may cause vaginitis. During the acute stage of the infection the gonococci probably invade the blood stream, but only in a few instances do the organisms survive or multiply sufficiently in the circulation to set up metastatic foci of the infection. The external genitalia are usually protected against the invasion of the organism by the thickly epithelized mucous membrane, and only in children or individuals with atrophic epithelium is there found a diffuse vulvo-vaginitis produced by the gonococcus. Because of the irritation of the gonorrheal discharge there may be seen the pointed or wart-like condylomas which are found around the vulva and the anus or on the penis, although they can not be regarded as a specific gonococcus granuloma. Occasional erosions around the orifice of the male urethra or a mild balanitis of the penis have been observed in which the gonococcus was the causal factor. The laboratory diagnosis of gonorrhea includes the demonstration and culture of the organism from the lesion and the demonstration of specific immune bodies by the complement fixation test.

Obtaining of Material. In males the examination is usually that of purulent discharge from an acute urethritis. In chronic inflammatory lesions the discharge may be scanty and only a small drop obtained on stripping the urethra or from prostatic massage. At times it may be necessary to examine mucous threads that may be found in the urine. In the female, smears are usually obtained from the urethra or the cervix. Smears from the cervix are made after first removing and discarding the plug of mucopurulent material from the orifice. Failure to find gonococci is often caused by inadequate methods of obtaining smears for examination and fresh material should always be obtained if possible.

Demonstration of Gonococci

The smears are stained with Gram's stain. Hucker's modification is especially adapted for the staining of gonococci and is recommended for this purpose.

The *Neisseria gonorrhoeae* is a Gram-negative diplococcus found intracellularly and extracellularly, the former being characteristic (see Fig. 18.8). It is a diplococcus with the adjacent sides flattened with the long axis vertical to the plane of junction. The organism measures approximately 1.6 microns in length and 0.6 of a micron in width and stains with many of the aqueous aniline dyes. In the presence of other

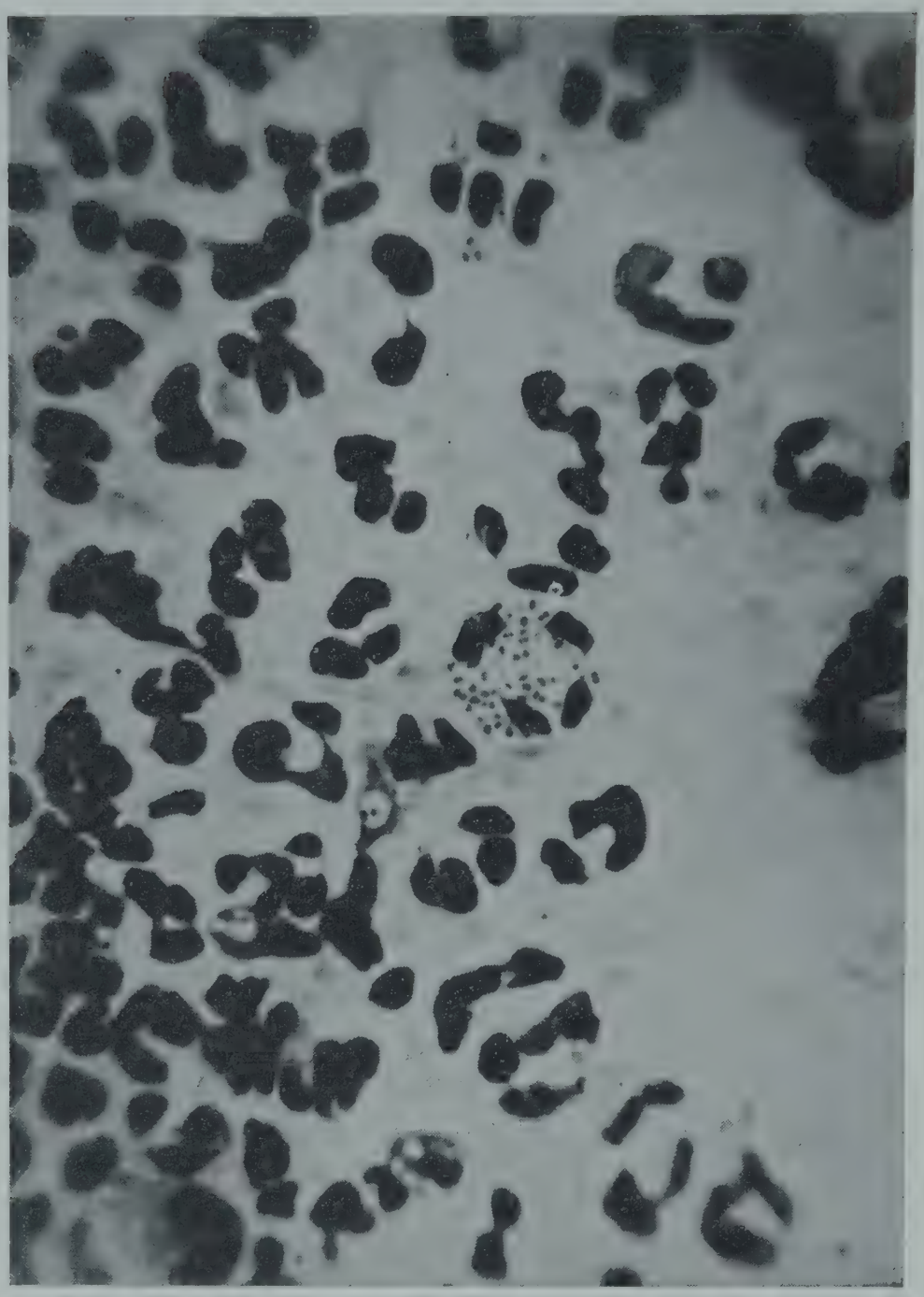


FIG. 18.8. Photomicrograph showing gonococci in a urethral smear

bacteria, gonococci are found with difficulty and in acute cases they usually appear extracellularly as well as intracellularly.

When such organisms are seen in urethral smears this is generally accepted as such strong evidence of gonococcic infection that the term "gonococci present" is used. However there are other cocci that have the same morphological characteristics, such as the *Micrococcus catarrhalis*. In cases of doubt, culture methods should be used.

Culture of Gonococci. The great diagnostic value of cultural methods for the demonstration of gonococci is now generally recognized. With some experience the cultural identification of the *N. gonorrhoeae* is not difficult. One of the conditions necessary for successful growth of the organism is that the time elapsing between the obtaining of the material and the preparation of the culture be as short as possible. Cox and McDermott suggest the following "shipping media" for preparing and

transporting infectious material to distant laboratories. To equal amounts of sterile distilled water and defibrinated horse blood a 1 per cent aqueous solution of crystal violet is added to make a dilution of the dye of 1:30,000. 5 mg. of para-amino-benzoic acid are added to every 100 ml. of the blood-dye mixture. Test tubes measuring 6 by 50 mm. are filled with 0.6 ml. of the thus prepared preservation fluid and stoppered with cork stoppers dipped in paraffin. The authors obtained 92 per cent positive results by culturing the material as late as three days after it was obtained.

The cultural method on ascitic fluid agar containing glucose, Vedder's starch medium, McLeod's chocolate blood agar, and Rosenow's brain broth are equally satisfactory. In all of these media the oxygen tension should be low, and this can be produced by replacing the air with carbon dioxide, by burning the available oxygen with a candle, or by reduction of the media through living tissue.

Probably the simplest method of growing the gonococcus is the use of Vedder's starch agar, described in 1915. It consists of beef infusion broth to which are added 1 per cent corn starch and 1.5 to 1.7 per cent agar. One part of meat is infused in two parts of water (distilled or tap) overnight. The meat broth is filtered until clear, then normal sodium hydroxide is added until it is 0.3 to 0.5 acid to phenolphthalein. After the broth has been adjusted to the proper acidity, 16 gm. of granular agar per 1000 ml. of broth are added. After this has dissolved, the starch mixed with a little water is added and the mixture stirred well. The media is then sterilized for 20 minutes at 15 lbs. pressure. Tubes or plates are poured in the usual manner and dried in an incubator until a slight splitting or shrinking of the media can be observed. Cultures are usually made with simple cotton swabs, which are inserted into the urethra or cervix.

By use of cultural methods it is possible to increase the number of cases in which gonococci can be demonstrated from 40 to 80 per cent, and it should be used as a routine procedure in the laboratory in those instances when the organisms can not be found in the direct smear.

The Complement Fixation Test for Gonorrhea

The presence of specific antibodies to gonorrheal infection was demonstrated experimentally by Brook in 1906 and applied for clinical purposes in the same year by Mueller and Oppenheim. A polyvalent gonococcus antigen and the use of the antisheep hemolytic system have proved to be the most satisfactory procedure in the United States. The antigen

may be purchased from biological supply houses (DiGioia) or may be prepared from fresh colonies of the gonococcus. The technic of the test employs Kolmer's modification of the Wassermann test except that the primary incubation period may be 15 to 18 hours in a refrigerator at 6 to 8°C. followed by one-half hour in the water bath or in a water bath alone for two hours. The most sensitive readings are obtained ten minutes after complete hemolysis of the antigen control in the case of those sera showing complete hemolysis of the serum controls. Otherwise it should be made immediately after the second incubation provided that the antigen-serum-hemolytic system and the negative serum controls show complete hemolysis.

In interpreting the test, it should be remembered that it becomes positive not earlier than two to three weeks after the infection has taken place. Simple cases of urethral gonorrhea often give a negative test, while cases with complications (arthritis, blood stream infections) or cases of chronic ascending gonorrhea (epididymitis) are more likely to be positive. Repeated negative tests over a period of three to four weeks make the presence of a chronic gonorrheal infection unlikely. The reaction is very specific and false positive results are obtained in less than 1 per cent. The complement fixation test remains positive for two to three months after clinical cure is established, and the change from a positive to a negative reaction can be taken as an indication of successful therapy. The reaction is particularly valuable in the diagnosis of chronic gonorrhea in the female (Jacobsen, Mason and Arnold), and it is not influenced by the medication of sulfanilamide (Cohn).

CHANCROID

Clinical Correlation. The Ducrey bacillus is responsible for many of the acute ulcerations and chronic granulomatous lesions on the external genitalia. In the male the clinical picture of soft chancroid in its typical forms is easily recognizable by the appearance of typical suppurative buboes which accompany the painful nonindurated ulcerations of the external genitalia. In the female the lesions are so variable that every case presents individual peculiarities.

The laboratory diagnosis of chancroid infection can be made in two ways: first, by the demonstration of the causal organism by smear or culture in the lesions or the regional lymph glands, and secondly, by a specific allergic reaction of the patient to the Ducrey bacillus by the intradermal test.

Collection of the Material. The Ducrey bacillus can be recovered

usually from very acute lesions with small ulcerations and little exudate. Later saprophytic organisms usually crowd the organisms out of the exudate and material from exudative ulcerative lesions is usually negative. In such instances the lesion can be covered for 24 hours with colloidion, which will kill most of the secondary invaders but does not affect the Ducrey bacillus.

It is known that the Ducrey bacillus may exist as a saprophyte in the female and male genitalia, and such persons may be infective carriers of the organisms. In cases of visible buboes the bacillus may be found in pure culture from puncture of the gland. It should be borne in mind that this infection may co-exist with syphilitic chancres, although this is an exceptional occurrence.

Demonstration of the Ducrey Bacillus

The organism of chancroid can be demonstrated in the slide stained with Gram's method in about 90 per cent of cases (Kolmer). The bacillus, described by Ducrey in 1889 (*Hemophilus ducreyi*), is a small, non-motile, Gram-negative rod measuring 0.5 to 1.5 micra in length. When stained with fuchsin the bacilli show polar staining. They are arranged in pairs, groups, or chains lying parallel to one another similar to the chains of streptococci (Fig. 18.9). Usually they are found extracellularly but may be intracellular. Sometimes they are difficult to find, especially in chronic lesions where mixed infection is present.

Culture of the Ducrey Bacillus. Cultivation of the Ducrey bacillus is difficult mainly because of its slow growth in a mixed culture. The media should be rich in blood and reduced oxygen tension seems absolutely necessary for successful growth.

Sanderson and Greenblatt recommend a media composed of 5 ml. of soft beef infusion agar (0.5 per cent agar) to which 1 ml. of human defibrinated blood is added just before inoculation. Not more than 0.2 ml. of aseptically aspirated bubo pus is added and the tubes are incubated at 37°C. for 2 to 3 days. When a positive culture is obtained it is necessary to transplant the colonies at three to four day intervals.

The Allergic Skin Reaction

This skin test for chancroidal infections is useful in chronic cases when the bacillus can not be demonstrated in the lesion. Described independently by Ito, Reenstierna and Dmelcos, it is quoted in the literature alternately as the Reenstierna or Dmelcos reaction. The bacillary antigen is prepared, according to Sanderson and Greenblatt,

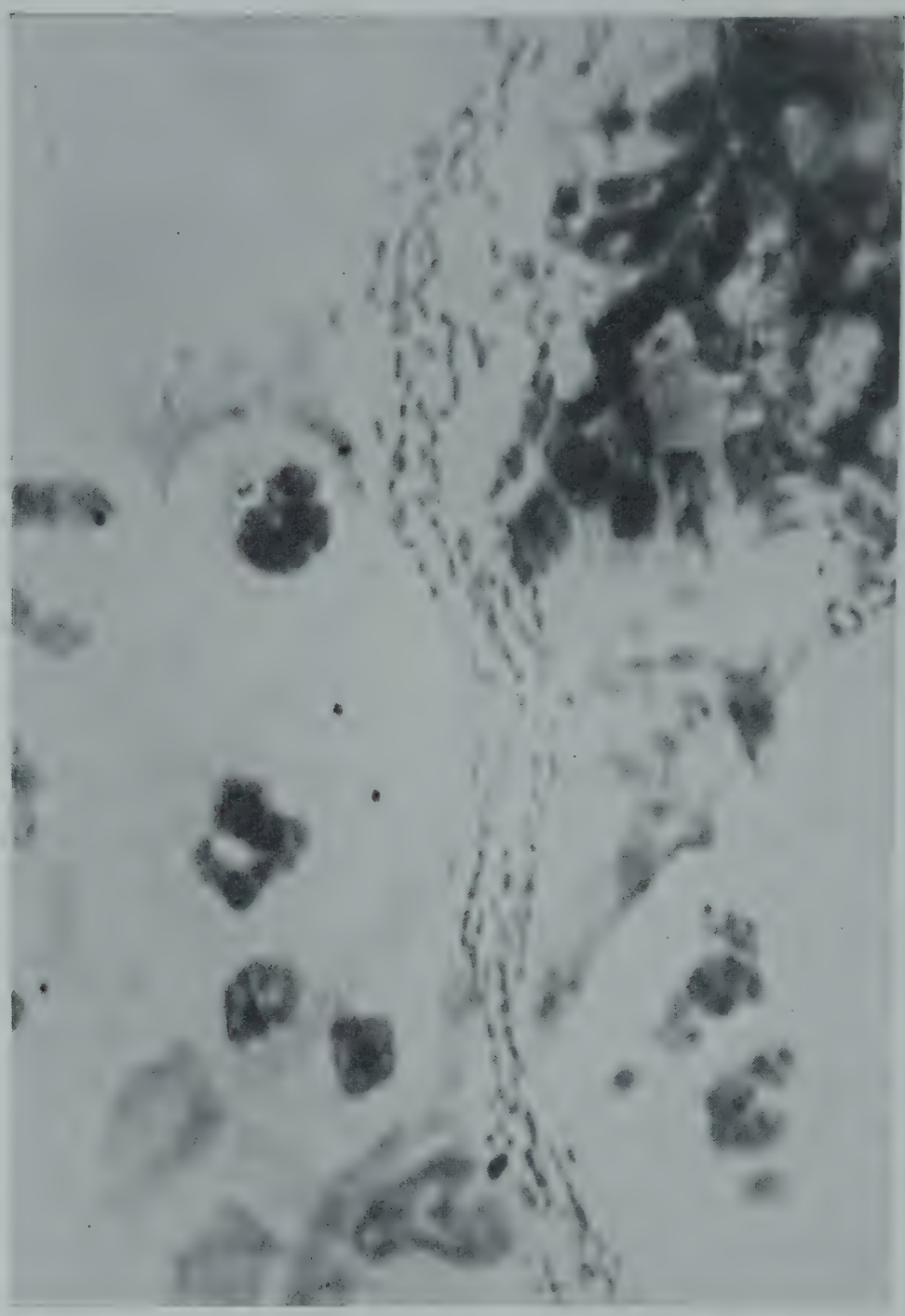


FIG. 18.9. Photomicrograph showing typical chains of Ducrey bacillus

from a stock culture transferred to the blood on the surface of a veal infusion agar slant. After two or three days of incubation at 37°C. the fluid blood is removed, diluted with water, and centrifuged at high speed. Two successive washings will remove most of the hemoglobin and serum, and the sediment containing the bacteria is suspended in 10 ml. of sterile saline solution and heated for one hour in a water bath at 60°C. A tissue antigen can be prepared from chancroidal pus diluted with saline solution in the proportion of 1:10 and heated for one hour at 60°C. The test is made by injecting 0.1 ml. of antigen intracutaneously, using for control a sterile saline solution. The reaction is read 48 hours later and may be graded from one to four plus. Reports from various investigators agree that the reaction is sufficiently specific and sensitive to be of considerable diagnostic value and that the bacillary antigen

recommended by Dmelcos gives more clear-cut results than the pus antigen recommended by Reenstierna (Greenblatt and Sanderson).

VENEREAL FUSOSPIROCHETOSIS

Clinical Correlation. The role of the fusospirochetal organisms in the production of pudendal lesions is now well recognized and the term "the fourth venereal disease" has been introduced by Corbus and Harris in the American literature for this type of infection. Fusospirochetal organisms may occur in the smegma of the penis and the clitoris of normal men and women and can be regarded in most cases as harmless saprophytes of the genital mucous membrane. They are capable however of producing painful, progressive and destructive ulcerations which lead to severe mutilation of the genitalia. In the male an extensive gangrenous balanitis with necrosis of penis and scrotum has been observed, while in the female the process may extend from the vulva into the vagina, which will take on a black gangrenous appearance (noma). While such extensive lesions may be regarded as rare, small superficial ulcerations are observed with increased frequency among the poorer population of the large cities in the temperate or tropic climates. The fusospirochetal organisms also may invade an existing venereal lesion and by active penetration may cause severe necrotic and gangrenous changes. Thus, fusospirochetal infection of a chancroidal ulcer can lead to severe phagedenic gangrene of the genitalia. Often the organisms can be found in the discharge of an already existing lesion, such as inguinal granuloma, without being able to penetrate into the vascular granulation tissues. Then their presence will only increase the odor and the amount of the discharge from the lesion. The differential diagnosis between primary and secondary fusospirochetosis of the genitalia is sometimes extremely difficult. Exclusion of all other possible etiological factors must be one of the main criteria in making the diagnosis.

Obtaining of Material. B. fusiformis and various spirochetes may be found in the smegma of the penis and vulva of normal men and women and can be regarded as saprophytes. They are most frequently found in the grayish exudate covering the shallow, confluent and painful ulcerations of primary fusospirochetosis. In the extensive gangrenous lesions, noma and phagedenic chancre, they may be missing in the superficial exudate but abundant in the necrotic tissue. Thorough cleaning with gauze and deep scraping of the lesion will be required to collect satisfactory material.

Demonstration of the Organism

Smear. The laboratory diagnosis of this infection is made by finding the fusospirochetal organisms in a smear from the lesion. The best method is the dark field examination, where the characteristic motility of the spirochetes and their shape and contour can be demonstrated. For the preparation of permanent slides the silver method of Fontana can be recommended, while a smear stained with Wright's stain may suffice for a more rapid diagnosis. As a simple working rule the spirochetes of the genitalia may be compared morphologically with those of the mouth, unless they have been proven different by culture. Thus the *Treponema genitalis* resembles the *Treponema microdentium*, the *Treponema accuminatum* resembles the *Treponema vincenti*, and the *Treponema phagedenis* and *balanitides* are very similar to the large *Treponema buccale*. These spirochetes are usually accompanied by the various types of fusiform bacilli, spirilla, vibrios, and cocci (Fig. 18.10).

With some experience it is possible to follow the course of the infection by studying the distribution and the amount of the bacterial flora observed in repeated smears. Spirochetes resembling *Treponema vincenti* associated with the thin short types of fusiform bacilli are usually found in rapidly progressive lesions (Fig. 18.10). Large spirochetes resembling the *Treponema buccale* and thick fusiform bacilli are usually found in the more chronic type of lesion. An increase in the fusiform bacilli usually precedes an advancement of the lesion. Under therapy the spirochetes will disappear first, while the fusiform bacilli will prove the more tenacious organisms.

Tissue. Silver impregnation of slides after the method of Levaditi and others will show an abundant amount of bacteria present with an invasion of the fusospirochetal organisms into apparently healthy tissue with the spirochetes further advanced than the rest of the symbiotic group.

The cultivation and isolation of the various members of this symbiotic group are extremely difficult and can not be recommended as a routine method. Animal experiments have failed to be of diagnostic aid.

GRANULOMA VENEREUM

Clinical Correlation. According to Manson's definition, granuloma venereum or granuloma inguinale represents an infectious granulomatous condition of the pudenda, widespread in some parts of the tropics and conveyed by sexual contact and autoinoculation.



FIG. 18.10. Photomicrograph showing *Treponema vincenti* from an acute fuso-spirochetal ulcer.

It is questionable whether or not granuloma inguinale represents a venereal infection. The clinical observations of Campbell, as well as the experimental work of de Monbreun and Goodpasture, make the traditional venereal nature of the disease doubtful.

The nodular form of the disease usually represents the early stages of the infection and appears as an elevated papule 1 to 4 cm. in diameter, reddish in color and of soft consistency. The most frequently observed lesion is the serpiginous ulcer. This develops from the primary nodule by excoriation of the superficial epithelium covering the papular lesion and the development of a rather spongy and easily bleeding granulation tissue which has the tendency to spread by continuity or contact auto-inoculation to the surrounding tissue.

Simultaneous with the progress of the lesion, scar formation which

usually starts in the center of the ulcer can be observed. The scar tissue is rather atrophic, shows pigment changes, and has an extremely avascular and dry appearance.

Obtaining of Material. Only during the early acute stage of the disease do we find the organisms in the superficial layers of the exudate. At any other stage they can be found in the granulation tissue, and material must be obtained by scraping the lesion with a sharp edged instrument. Local anesthesia of the surface is recommended to prevent pain. Care has to be taken that the scraping knife penetrates through the necrotic and secondarily infected surface of the ulceration in order to obtain a smear from the deeper layers of the granulation tissue. Repeated examinations are always required and a single negative smear never excludes the presence of the disease.

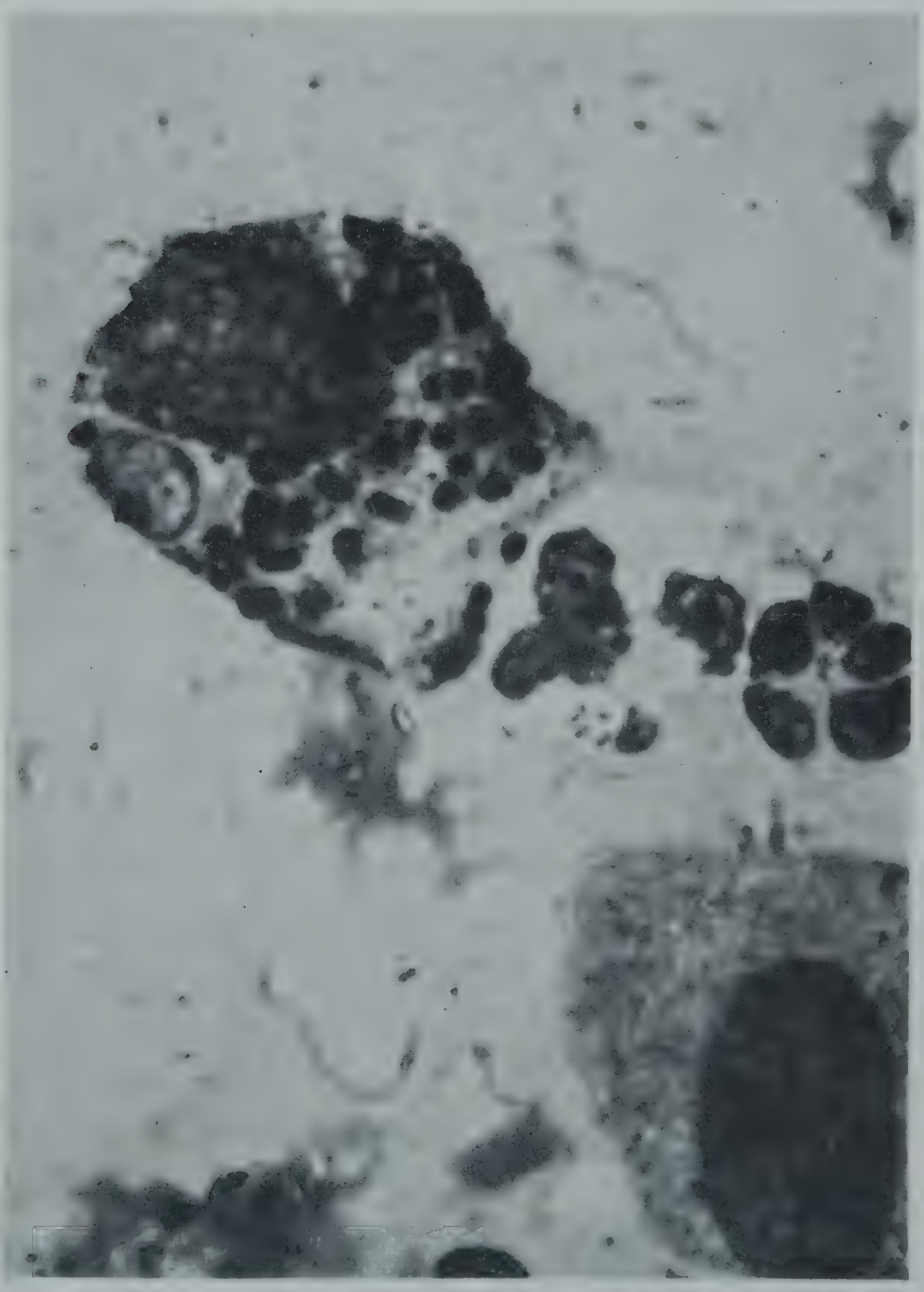


FIG. 18.11. Photomicrograph of the encapsulated Donovan bodies in the smear from a chronic lesion.

Demonstration of the Causal Organism

In the Smear. The demonstration of the characteristic Donovan bodies in the lesion or the discharge is the all-important laboratory method of diagnosis. They can be found in smears from the lesion stained by Wright's method in 60 to 80 per cent of cases. The organisms appear in the stained smears as small encapsulated bodies (Fig. 18.11) the nucleus of which resembles a small curved bacillus showing one or two terminal swellings simulating polar bodies (diplococcoid bodies of Randall). In very acute and fulminating lesions these organisms are not encapsulated and can be recognized extra- and intracellularly (Fig. 18.12).

In Tissue. A much higher per cent of successful demonstration of the organisms can be obtained in tissue sections. Margolis recommends

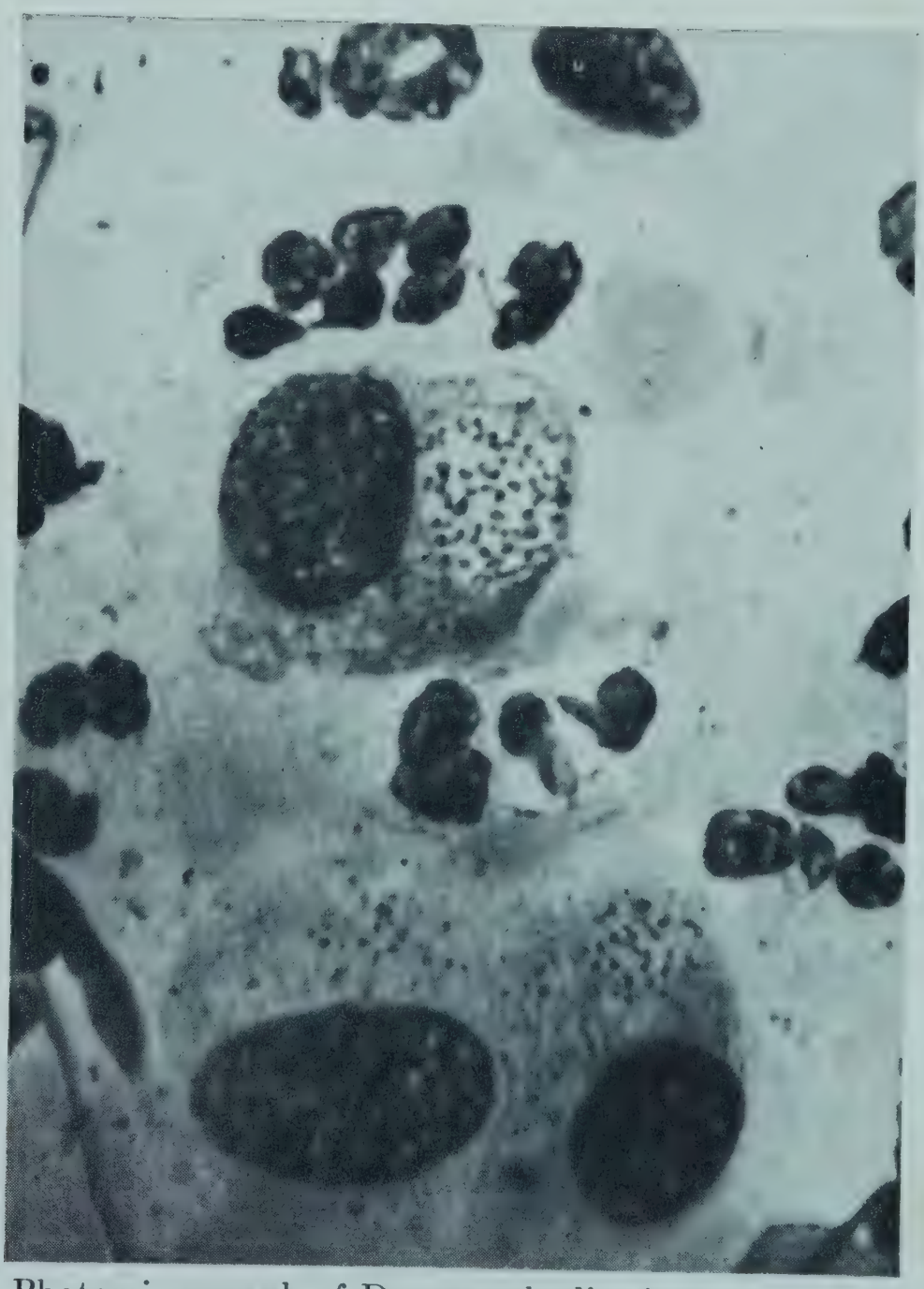


FIG. 18.12. Photomicrograph of Donovan bodies in the smear from an acute lesion.

the staining of frozen sections with Terry's neutralized polychrome methylene blue. Sections are cut 20–25 micra in thickness and dipped in the dye for a few seconds. Large parasitized multinuclear cells can be readily recognized under high power magnification. The Donovan bodies stain a delicate blue. The capsule is clear. The organisms measure 0.6 to 0.8 micra in length and 0.6 to 0.8 micra in diameter. Alexander and Schoch recommend staining of the sections with Giemsa stain. We have had equally good success with the Wright method or with a 1:1000 solution of cresyl violet. The organisms can be found intracellularly in the large endothelial cells and histiocytes in the deeper layers of the infected tissues. It also can be found in the hypertrophic and fibrous type of lesion where the absence of discharge would make the diagnosis otherwise impossible. In these cases only large encapsulated forms can be demonstrated.

Histopathology

The importance of the biopsy in the diagnosis of this disease has been stressed by many authors (Brandt and Gatewood). In the very early or nodular type of granuloma venereum the epithelial lining of the skin shows acanthosis, edema and spongiosis. The corium is infiltrated with polymorphonuclear leukocytes, eosinophils and large monocytes (endothelial cells). Rapid proliferation of capillaries in the area of infiltration marks the beginning of the development of granulation tissue, which soon breaks through the epithelial lining of the skin to form the typical serpiginous ulcers. Plasma cells, diffuse and in small groups, become increasingly prominent with progression of the lesion, and the leukocytes, which previously seemed to be the important primary cellular response, are now found only at the surface. Large endothelial cells with numerous intracellular Donovan organisms are profuse between the capillary loops of the granulation tissue and are probably identical with the large foam cells described by Goldzieher and Peck as characteristic of this stage of the disease.

As the healing progresses, fibrocytes, which primarily were only sparsely scattered between the capillaries of the granulation tissue become more abundant and, from the epithelial islands which have remained intact during the process of granulation tissue formation, re-epithelialization of the surface begins. The scar tissue that repairs the serpiginous ulcer of granuloma inguinale usually shows a narrow epithelial lining with loss of all special structures of the skin and a moderate degree of subepithelial fibrosis. Histological examination of the hyper-

trophic and the keloid-like lesions reveals marked fibrosis with numerous small nests of plasma cells and endothelial cells. The epithelium appears normal in thickness or shows slight hyperplasia. The walls of the larger vessels are thickened and their lumens narrowed. The collagenous substance is extremely abundant. In the small collections of inflammatory cells Donovan bodies can be found, thus giving evidence of the activity of the lesion.

LYMPHOPATHIA VENEREUM

Clinical Correlation. Venereal lymphogranuloma is an infectious disease caused by a filtrable virus, and is characterized by a variety of clinical manifestations. It is principally a disease of the lymph vessels and the lymph glands of the pudenda, and the difference in the manifestations between the male and the female can be explained by the different anatomical relationships of the sexes. The acute stage of venereal lymphogranuloma is characterized by the appearance of a small, usually negligible primary lesion followed by a prolonged suppurative process in the regional lymph glands. The chronic stage of the disease is characterized by a slowly spreading lymphangitis with lymph vessel obliteration and subsequent lymphedema of the affected parts leading to elephantiasis and trophic ulcerations (esthiomène).

The laboratory has developed two very reliable means of diagnosing venereal lymphogranuloma: the demonstration of the virus in the diseased tissue, and the specific skin reaction of Frei.

The Isolation of the Virus

The virus can be isolated from the primary lesion or the bubo, and the discharge from these lesions or the tissue itself can be used for the isolation. Most suitable for the demonstration of the virus is the aseptically excised or aspirated bubo in the acute stage of the disease. One-tenth of a ml. of the pus, which has been diluted with equal parts of sterile physiological saline solution, is injected intracerebrally into six white mice. The animals usually die within three to five days after onset of the symptoms, and histological studies of the brain reveal an extremely cellular exudate consisting of lymphocytes and large endothelial cells around the vessels of the meninges (Fig. 18.13).

The antigenic property of pus or tissue from venereal lymphogranuloma can be demonstrated by preparation of a 20 per cent emulsion of the material with physiological saline solution, heating on two successive days for two hours at 50–60°C. and testing it against a patient with known

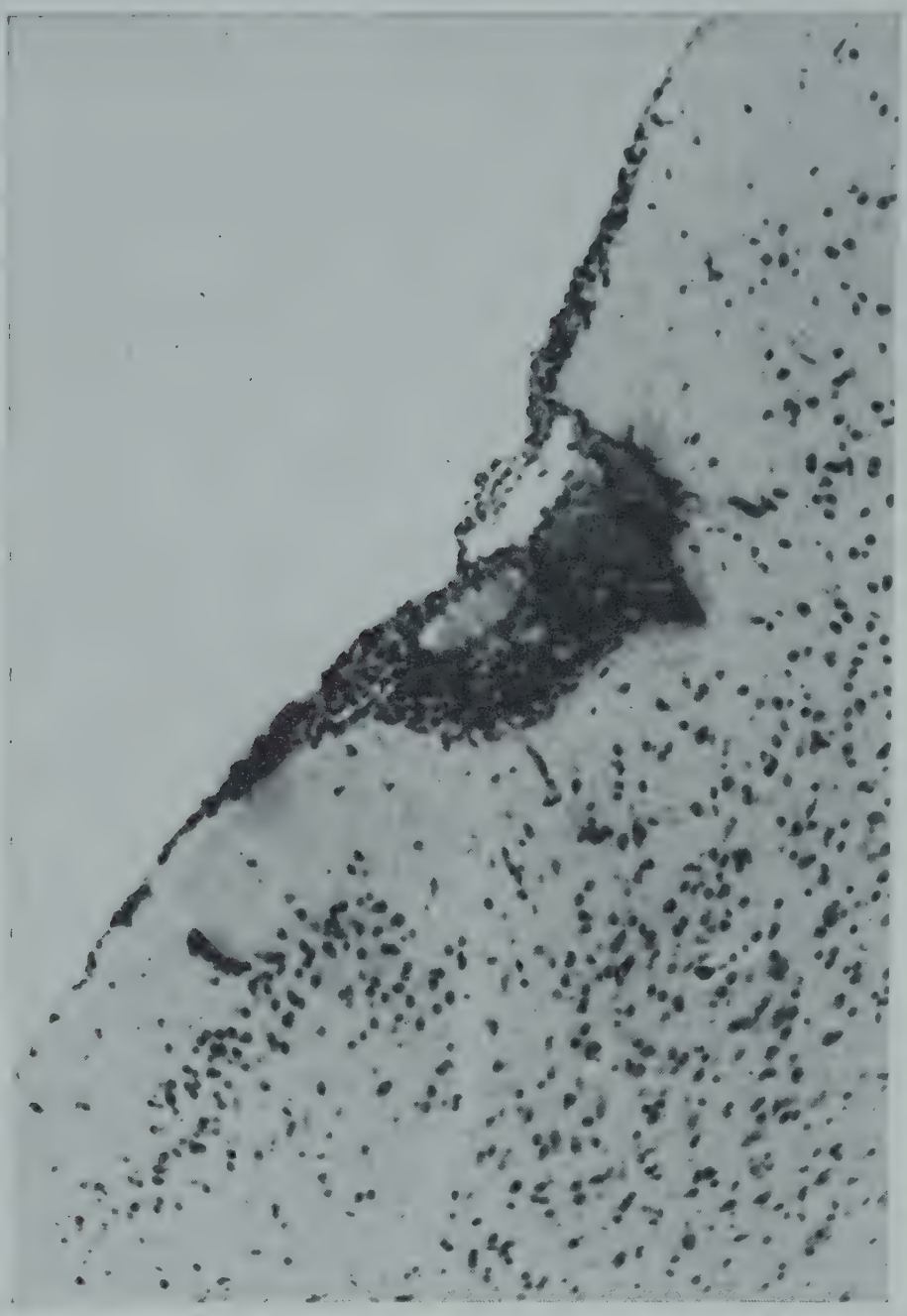


FIG. 18.13. Photomicrograph of the brain lesion produced by the virus of venereal lymphogranuloma in the infected animal.

history of the disease. If a positive skin reaction is obtained, it can be taken as unmistakable proof that the tested emulsion contains the specific antibodies of venereal lymphogranuloma.

The Complement Fixation Test

Yolk sac antigen prepared from infected eggs proved to be a very satisfactory antigen and has increased considerably the sensitivity of the complement fixation reaction (Shaffer, Rake and Grace). Positive reactions were obtained as early as seven days after the onset of symptoms. Commercially available antigens (Lygranum-Lederle) are suitable for use as antigen for the test. For the performance of the test the following reagents are added to the tube in amounts of 0.2 ml. of each and in the stated order: patient's serum inactivated at 56°C. for one half hour and diluted 1:2 or more, 2 hemolytic units of guinea pig complement and

antigen. After allowing the mixture to stand 75 minutes at 37°C., 0.2 ml. of a 3 per cent sensitized sheep corpuscle suspension is added and readings are made after a further 30 minutes at 37°C. Adequate controls include a tube with antigen prepared from non-infected yolk sacs in a manner similar to that of the specific antigen. This control antigen is also commercially available. In interpreting the reaction the titer of the unknown serum is the highest dilution of the serum showing complete or nearly complete fixation of the lygranum antigen and no fixation or only a trace with the control antigen. Since with this method the serum is at least diluted three-fold the lowest possible titer which can be observed is 1:3. Titers up to 1:600 have been observed. Grace and Rake claim that the complement fixation test for lymphopathia venereum is more sensitive than the allergic skin reaction of Frei and that a definite relationship exists between the average titer of lymphogranulomatous serum and the average dimensions of the Frei reaction. Prolonged therapy with sulfonamide compounds does not remove the complement fixing antibodies from the serum of lymphogranulomatous patients. Levine and coworkers claim cross complement fixation between antibodies of lymphopathia venereum and psittacosis. It is evident that more data have yet to be accumulated to establish this test firmly in the diagnosis of lymphopathia venereum.

The Specific Skin Test

Introduction of the specific skin test by Frei in 1925 in the diagnosis of venereal lymphogranuloma has facilitated the diagnosis of this disease and aided in establishing the complete clinical entity of the infection.

Antigen prepared from the yolk sac of infected eggs is now commercially available and is best suited for the performance of the Frei test.

In the application of the skin test certain precautions should be observed. It is important to inject the material intradermally and for this purpose a 27 gauge needle and tuberculin syringe should be used. If properly injected, 0.1 ml. will produce a wheal of 6–7 mm. diameter. No bleeding should follow the injection. The hairless flexor surface of the forearm should be used, and the control injection should be at least 10 cm. from the test. The skin test is read 48 hours after the injection. In a positive case there will be an inflamed elevated area at least 7 mm. in diameter (Fig. 18.14). This area may be slightly painful, indurated, and may show a small punctiform abscess in the center of the



FIG. 18.14. Positive Frei reaction

lesion. The symptom of induration in a positive Frei reaction is more significant than the red color of the reaction since it is possible to distinguish this reaction even in very dark Negro skins. In negative cases complete absorption of the injected material takes place and only a minute induration of the size of a pinpoint is seen. A positive Frei reaction remains visible for 8 to 10 days, and after its disappearance there may often remain a permanent pigmented area.

Concerning the evaluation of the Frei test in diagnosis of the disease, the papers by Hellerstroem and Wassen and D'Aunoy and von Haam may be consulted. The specific immunity produced by infection with the virus of venereal lymphogranuloma is of long duration and perhaps permanent, and this is important in the evaluation of the test. Just as in the tuberculin reaction, a negative test is of more diagnostic value with regard to the present lesion than a positive test, which never

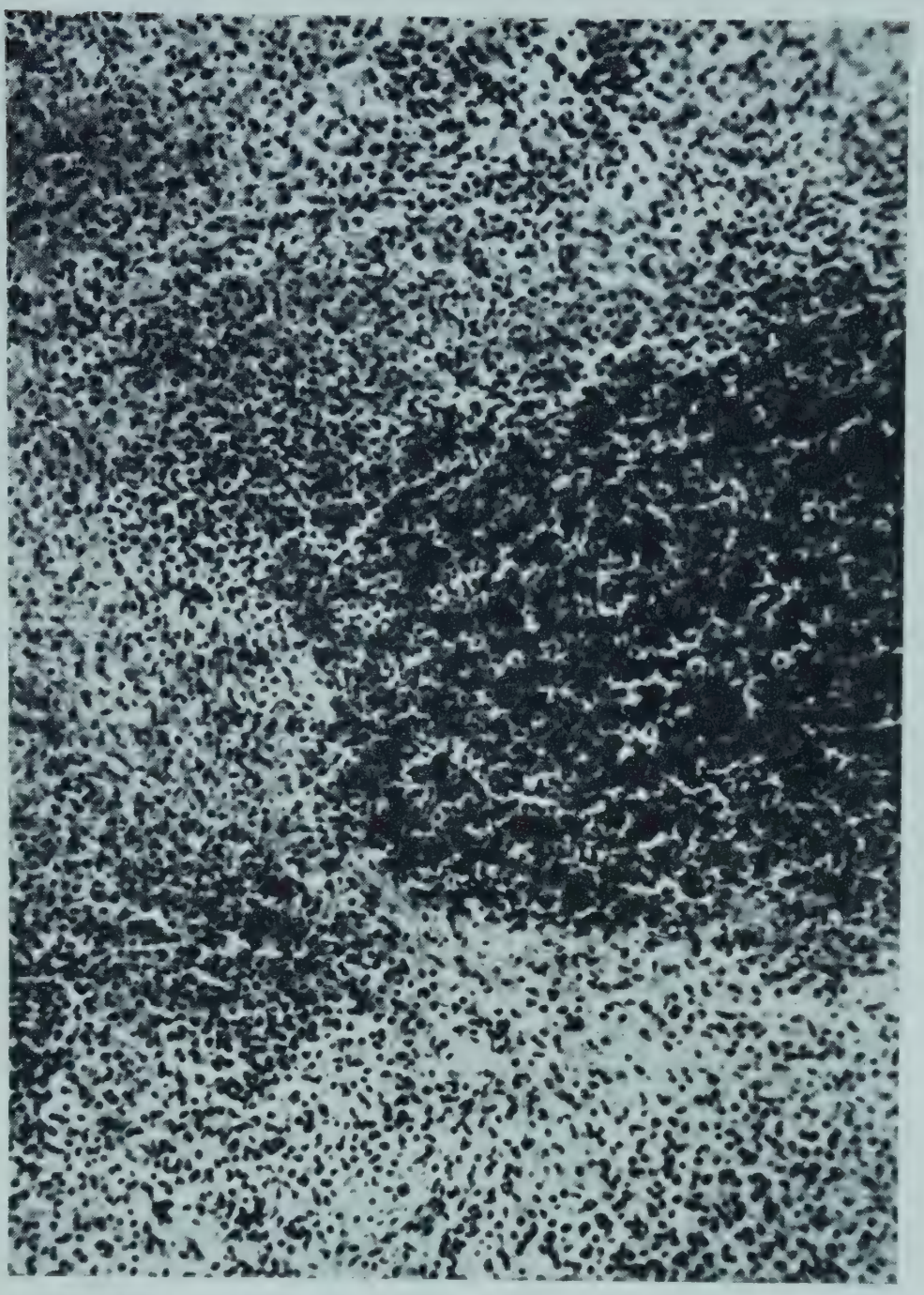


FIG. 18.15. Photomicrograph of abscess formed in inguinal bubo

excludes a previous infection. Only during pregnancy, in long and exhaustive diseases, and in the acute stage of secondary syphilis can a transient disappearance of the specific immunity be observed.

The Histopathological Diagnosis

The most typical histological picture characteristic of the disease is seen in the buboes, where marked proliferation of the endothelial cells and the collection of large mononuclear cells in the form of small nodules is the first evidence of the infection. Later, necrosis occurs in the center of these nodules with invasion of polymorphonuclear leukocytes producing microscopic abscesses which usually assume a triangular or quadrangular shape (stellate abscesses) (Fig. 18.15). Histological examination of the chronic manifestations gives a less characteristic picture, and the diagnosis can only be suspected from the microscopic examina-

tion. Centering of the inflammatory process around markedly dilated lymph vessels with peri- and endo-lymphangitis is the most characteristic change.

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CHAPTER 19

SYPHILIS SEROLOGY

By Seward E. Miller

GENERAL CONSIDERATIONS

Historical

The serologic diagnosis of syphilis originated in 1906 when Wassermann and his colleagues, applying the principle of complement fixation, developed a serologic test for syphilis. At first this new test was believed to be specific for syphilis although generally used by physicians only to confirm a clinical diagnosis.

In a disease as chronic and protean as syphilis it has been only natural for physicians to turn to the laboratories hoping for a foolproof scientific tool and guide in its diagnosis. The laboratory scientists have responded enthusiastically, and during the past 49 years there has been constant effort to develop more sensitive and more specific serologic tests with the objective of detecting all syphilitic infections but not yielding positive results in other conditions.

As laboratory performance was improved physicians gradually developed strong reliance on these serologic reactions until in all too many minds they supplanted all other information as the basis for establishing a diagnosis of syphilis. During the past two decades increasing awareness of the limitation of these tests and the recognition of the occurrence of the so-called "biologic false positive" serologic reaction have resulted in a more moderated concept. Now it is generally recognized that the finding of a serum positive even by a number of tests does not prove the presence of syphilitic infection, while a negative serologic reaction does not necessarily rule out the existence of a syphilitic infection.

Types of Tests

The purpose of all serologic tests for syphilis is to detect in the blood of patients with syphilis antibody-like substances known as reagins. Although reagins are not true antibodies, they have some features in common with them. The various serologic tests developed for the detection of these reagins are of two types. One is based on the principle of complement fixation; a second, on the principle of flocculation. A third type, detecting what may be true syphilitic antibodies, utilizing

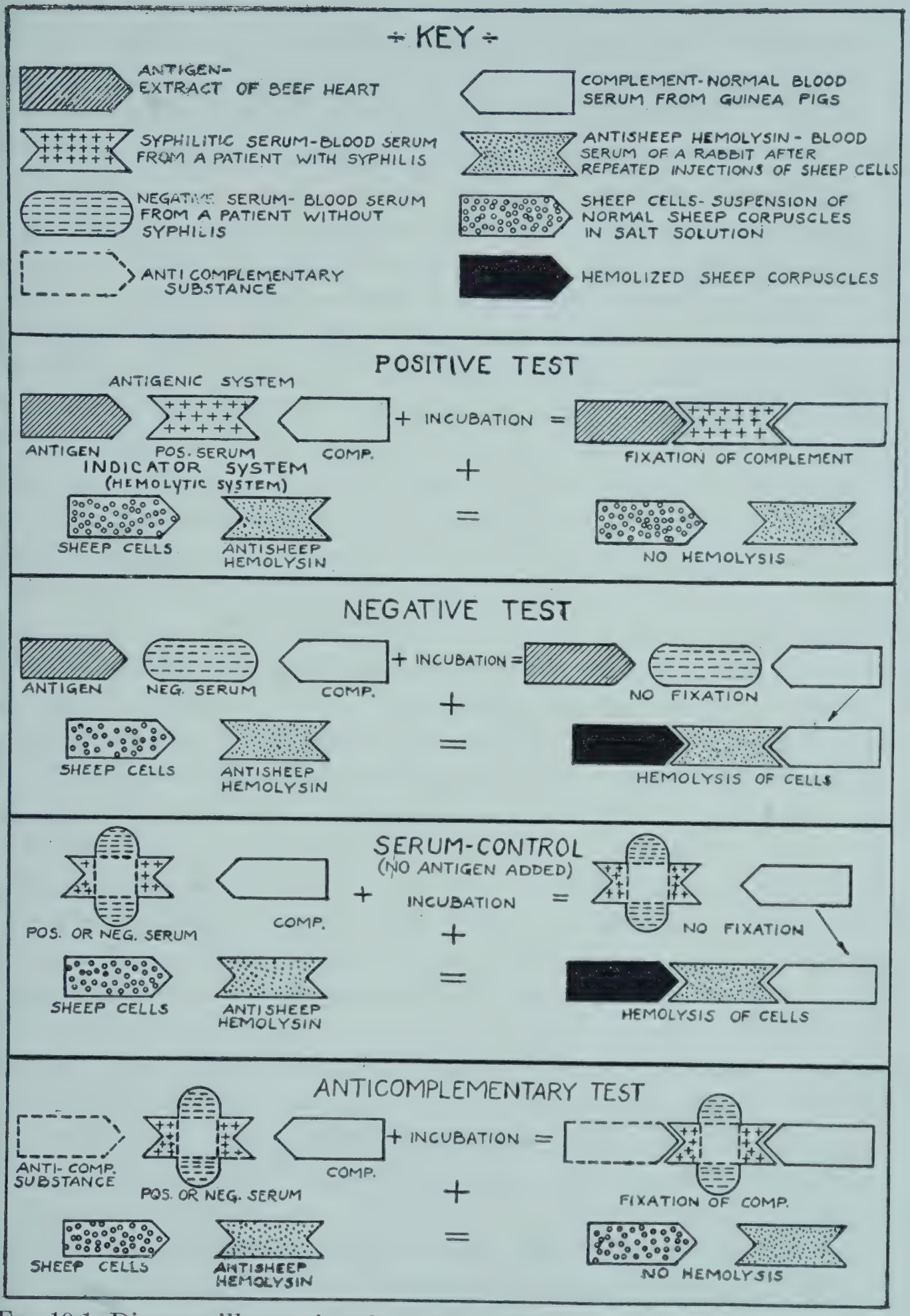


FIG. 19.1. Diagram illustrating the action of the various reagents used in the complement fixation test.

T. pallidum organisms, is based upon immobilization and killing of the *living* organism or agglutination of the *dead* organisms.

COMPLEMENT FIXATION TESTS FOR SYPHILIS

The complement fixation tests now in use are modifications of the original Wassermann test.

In all complement fixation tests for syphilis a so-called "antigen" is used. This is made of an extract of beef heart, or of cardiolipin, the purified active substance, to which cholesterol and lecithin are added. The antigen, suitably diluted with physiologic salt solution, is added to the inactivated serum of the patient, and this is followed by addition of normal serum of guinea pigs (complement). The mixture is incubated at 38°C. If the patient has syphilis, the reagin in this serum combines with the antigen. The union of the antigen with antibody uses up or fixes the available complement, hence the name complement fixation tests. This fixation is a quantitative reaction depending upon the quantitative relations of the antigen-antibody union. Since the fixation of the complement is an invisible reaction, it is necessary to introduce an indicator in the form of a so-called hemolytic system; for instance, an antisheep hemolysis and sheep red corpuscles. If syphilitic reagins are present, their combination with the antigen fixes the complement and no hemolysis of the sheep cells occurs, and the test is positive. In the absence of reagins, complement is free to hemolyze the sheep cells sensitized by the antisheep hemolysis and the test is negative. There are many modifications of the original Wassermann technique. They differ considerably in sensitivity, i.e., the ability to detect small amounts of syphilitic reagins, and in specificity, i.e., the freedom from false positive reactions. It is essential, therefore, to refer to them by the names of their authors, for example, the Kolmer or the Eagle modification of the complement fixation test instead of using the general term "Wassermann Test." These methods are rather complex with so many variable factors that the accuracy of results from different laboratories is usually proportional to the experience and knowledge of the serologist who performs the test.

In the Kolmer modification two methods are available. One, the quantitative test, employs five graduated doses of serum or spinal fluid and is generally preferred, especially in testing the serums from cases of syphilis under treatment in order to secure serologic evidence of improvement. The second, designated as the qualitative test, uses 0.2 ml. of serum, with an additional tube as a control (0.2 ml.) and is sufficient for diagnostic purposes as well as being more economical of ma-

terials and time required. Both are of equal sensitiveness and specificity. The test may be conducted also with a single dose of 0.1 ml. of serum with 0.1 ml. in the serum control although the reactions are not quite as sensitive as tests employing 0.2 ml. of serum.

Sources of Errors

Defective Complement. In the great majority of instances difficulties are due to complement supersensitive to the anticomplementary effects of antigen, serum, or both. This is especially likely to occur during the hot months of the year. Sometimes complement may be defective in hemolytic activity and whenever the unit is higher than 0.5 ml. of 1:30 dilution it should not be used. Occasionally preserved complement is troublesome.

Defective Hemolysin. This is probably least likely to cause trouble although usually first suspected, especially if the hemolysin has been previously used with success. The unit of antisheep hemolysin should be at least 0.5 ml. of 1:4000 and serums of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, hemolysin is rarely responsible even when shipped over long distances.

Defective Corpuscles. When blood is obtained from an abattoir one may encounter corpuscles possessing increased resistance to serum hemolysis. The cause of this rare phenomenon is unknown. A fresh supply of blood is the best remedy.

Anticomplementary Antigen. If no mistakes have occurred in dilution and dosage, this is very rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is, as a rule, due to some component of the hemolytic system, especially the complement.

Anticomplementary Serums and Spinal Fluids. Anticomplementary serums and spinal fluids will show incomplete hemolysis of the serum and spinal fluid controls. Such tests must be repeated with fresh serum and spinal fluid. Sometimes the majority of serums in a test show incomplete hemolysis of the controls. This trouble is due mainly to defective supersensitive complement. For this reason the unused portions of all serums and spinal fluids should be kept in the refrigerator until the tests are completed in case it is necessary to repeat the test.

FLOCCULATION TESTS FOR SYPHILIS

When a properly prepared lipid extract of beef heart, the so-called "antigen," suitably diluted with physiologic saline, is mixed in certain proportions with inactivated syphilitic serum, a precipitate is formed.

This is the underlying principle of all flocculation tests for syphilis. They do not require complement or a hemolytic system and take considerably less time to perform. The better known are the VDRL, the Kahn, the Kline, the Eagle, the Hinton, and the Mazzini tests. These flocculation tests are of two types—those performed in test tubes such as the Kahn, VDRL tube, Eagle and Hinton, and those performed on glass slides such as the Kline, VDRL slide, and Mazzini. The latter or slide tests are frequently referred to as microflocculation tests. In the tube tests specified amounts of inactivated serum and properly prepared antigen are mixed *in the test tubes*, agitated, and then examined for precipitate. In the slide tests the components are pipetted directly onto specifically prepared slides and then mixed by agitation. The agitation for most tube tests is by a shaking machine but the Eagle test requires centrifuging. The microflocculation or slide tests are usually agitated on a mechanical rotator but either the slide or tube test generally can be satisfactorily agitated by hand, except, of course, the Eagle test. The tube tests are generally read by direct observation for precipitate while the microflocculation or slide tests are examined microscopically.

TREPONEMA PALLIDUM IMMOBILIZATION TEST (TPI TEST)

The treponemes used as antigen for the test are obtained from rabbit testicular syphilomata. The patient's serum is inactivated as for other serologic tests and is mixed with the suspension of motile treponemes in a test tube. A small amount of complement is added and the test is incubated for 16 hours at 35°C. in an anerobic jar filled with 5 per cent carbon dioxide and 95 per cent nitrogen. Samples from the tubes then are examined under a dark field microscope. When treponemal immobilizing antibody is present the treponemes are non-motile in the tubes containing complement but are motile in the control tubes. The antibodies demonstrated by this test have been shown to be distinct from reagin and probably are true syphilitic antibodies.

THE PROZONE PHENOMENON

Some of the early work in immunology on the agglutination of bacteria by sera demonstrated clearly that there are certain optimal ratios of serum to bacteria for this phenomenon. This failure to agglutinate bacteria in concentration was designated by Ehrlich as "the proagglutinoid" zone. In syphilis serology there is also an optimal zone or ratio for reaction between antigen and reagin. This is true in both the com-

plement fixation and flocculation tests, but it is much more prominent in the flocculation tests. A positive serum can be easily diluted to the point where there is insufficient reagin remaining to give a positive reaction. On the other hand as the amount of reagin in a serum increases its reaction with the same optimal amount of antigen becomes weaker and weaker until finally no reaction occurs. This is the so-called prozone reaction. It has been pictured as an excessive amount of reagin overwhelming a small amount of antigen. Among the flocculation tests those tests employing antigen ratios geared to detect the smallest amounts of reagin generally show the more pronounced prozone reactions. Thus, the more sensitive the flocculation test, the more susceptible it is to prozone reactions. The highest amounts of reagin are found generally in acute secondary syphilis and some cases of active tertiary syphilis. The incidence of prozone reactions has varied in different studies from 0.1 to 0.3 per cent.

Various devices are utilized in different laboratories to overcome this difficulty and to avoid the serious error of submitting a completely negative report on a serum that actually contains great amounts of reagin from a case of very active syphilis. Many laboratories perform more than one test on each serum submitted and routinely use a complement fixation test as one of these tests. Other laboratories for various reasons may prefer to run only flocculation tests but utilize at least two tests employing relatively different antigen serum ratios. Many laboratories employ routinely a very sensitive screen test and then examine all the positive of doubtful sera by one or more of the tests which are less sensitive but more highly specific. Then finally more and more laboratories are taking all the weakly reacting serums, the doubtfuls, and those serums having a "rough" appearance, diluting them and performing a quantitative test on them. In this way the prozone phenomena is obviated. Unfortunately, some laboratories less alert to this problem do little on their own initiative to safeguard against it. Moreover, in spite of the best safeguards, cases of very active syphilis may rarely have their serology reported as negative. Therefore, a physician must always be cognizant of this phenomenon and on the alert to request a quantitative examination of the serum in such cases.

REPORTING OF RESULTS

All syphilis serologic tests originally were reported either as negative or ± (plus-minus doubtful), or positive 1+, 2+, 3+, or 4+. Some schemes of reporting did not actually use the words doubtful or positive,

using only negative, ±, 1+, 2+, 3+, or 4+. Others, using the adjectives, included the 1+ and/or 2+ results with the doubtful adjectives and the 3+ and 4+ with the word positive, but always included the numerical reading. This pseudo-quantitative reading of a strictly qualitative test implied strength of titer, and was often erroneously interpreted by many physicians. Also, this system lead to the unfortunate unit system of reporting quantitative titers in positive serums. This was based on the fact that a 4+ reaction was accorded 4 units. All such systems tend to magnify the inherent errors in such reporting. Therefore, in recent years most syphilologists and serologists have agreed upon reporting of all qualitative test reactions as positive, doubtful, or negative, and *discarding* all numerical figures in such reporting. With the advent of present-day rapid treatment methods quantitative serology reports are essential in following the efficacy of therapy. In order to effect a uniform system for comparison of cases, the majority of laboratories now have agreed upon reporting quantitative tests by the greatest dilution at which the tested serum produced at positive reaction. Thus the serum or spinal fluid is diluted with saline 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, etc. The highest dilution giving a positive reaction is reported as positive—1:32, for example. The serum which gave a positive reaction only in the undiluted state is reported as positive 1:1. Harris has suggested that positive quantitative reports be recorded to show the highest dilution at which a positive reaction occurs and that the term "dils"—a contraction of the word "dilutions"—be used. For example, a serum giving a positive reaction at dilution 1:32 but not at 1:64 would be reported as positive 32 dils. Under this system a serum reading positively only in the undiluted state would be reported as positive 1 dil.

Serum dilution end points of positive reactions are commonly used in reporting agglutination tests for typhoid, paratyphoid, typhus, and other infectious diseases. Many people are familiar with this and to standardize quantitative serological reporting all quantitative syphilis serology should be so reported. It would appear of no practical significance whether the term dils is used or not.

The important thing about reporting of serologic results is that in order to be of most value to the physician all tests reported as positive should be accompanied by a quantitative report.

BASIC PRINCIPLES

All the complement fixation and flocculation techniques now in use are quite empirical. The reagin detected by these tests while bearing

some definite connection to syphilitic infection is not believed to represent immune bodies. The antigens used are made from normal mammalian (usually bovine) heart muscle. Despite all the technical advances made these tests remain empirical. With all the recognized limitations inherent in such a situation indeed the surprising fact is that the tests have been so successful.

A large part of this success can be attributed to strict adherence to standard, widely-used techniques; to a nation-wide intra-laboratory standardization program to maintain high standards of test performance; and to care in the mechanics of taking and handling of the blood specimen.

COLLECTION AND HANDLING OF SPECIMENS

In order to obtain serum clear and free from chyle, blood specimens should be withdrawn before meals and all equipment used should be chemically clean and dry. If the specimen is to be mailed the equipment should be sterile. The skin over the vein to be punctured should be appropriately cleaned and then allowed to become *perfectly dry* before insertion of the needle. The needle should be at least 20 gage and if a syringe is used the needle should be detached before gently transferring the blood to the test tube. The test tube should stand upright for 30 minutes undisturbed, then the blood specimen placed in an ice box. When these precautions are used, clear serum relatively uncontaminated and without hemolysis or chyle can be obtained.

ANTIGENS

As previously pointed out, all the commonly employed antigens are alcoholic extracts of beef heart which are fortified by the addition of cholesterol and in some cases other lipid substances. Each lot of antigen must be adjusted to the predetermined level of sensitivity decided upon by the author of each particular test. Specific antigens prepared from various strains of cultured non-pathogenic spirochetes have been prepared, but so far these antigens have not proven as reliable as the non-specific beef heart antigens.

Over a long period of time there have been recurrent efforts to obtain the active principle of these beef heart antigens in pure chemical form. Pangborn has isolated and purified a non-nitrogenous phospholipid, the active principle of the beef heart extracts originally used in preparation of "antigen." The sodium salt is characterized as having a mol. wt. of 2195 and iodine number of 120–125. The calculated formula is $C_{120}H_{208}O_{24}P_3Na_3$. Pangborn has designated this product as cardiolipin.

When properly mixed with lecithin and cholesterol there result antigens that are chemically reproducible and which exhibit highly specific complement fixing and flocculating activities with syphilitic serums. Different tests require different proportions of cardiolipin and lecithin for optimal results. It was hoped that such antigens would prove specific in reacting only with syphilitic reagin. Wide experience has shown these antigens to be not altogether specific in that positive reactions are obtained in cases of leprosy and a few other diseases. However, particularly in malaria and some other diseases, there is a marked reduction in the number of serums giving a positive reaction. At the same time when optimal proportions of cardiolipin, lecithin and cholesterol are used the tests are more sensitive than with older antigens. So uniformly promising have all the studies with cardiolipin antigen been that there is now hope for bringing about some agreement upon a single standard blood test for syphilis. To expedite this hope, the Coordinating Committee on Laboratory Methods of the American Public Health Association has developed a microflocculation reference standard test for syphilis using cardiolipin antigen.

SUPERSENSITIVE AND SCREEN TESTS

Since the sensitivity of any test can be increased by simply increasing the sensitivity of the antigen, Kahn devised the Kahn presumptive and Kline, the Kline exclusion test as supersensitive tests for detecting small amounts of reagin. However, the great difficulty with these and all similar tests is that they also give more false positive reactions. Such tests, however, have a useful purpose as screen tests. In practice a laboratory using a supersensitive screen test generally reports immediately only the negative screen test specimens. All specimens showing any reactions are then retested by one or more of the less sensitive but more highly specific procedures, and only the latter reactions are reported. Such systems are used profitably by laboratories having large numbers of negative specimens with only a relatively few positive specimens.

EVALUATION STUDIES

During the first two decades following the introduction of serologic tests for syphlis, the status of syphilis serology was truly chaotic. A multitude of tests had been introduced. Most laboratories were "playing" with the various tests, using their own short cuts or pet versions of the author's original technique. Thus great variations of sensitivity

and specificity existed between laboratories and frequently results were far from uniform in the same laboratory on different days. The Health Organization of the League of Nations early recognized this condition and in an effort to standardize performance invited the leading serologists to complete with their test in evaluation studies held in Copenhagen in 1928 and Montevideo in 1931. Since 1937 the U. S. Public Health Service with the cooperation of the American Society of Clinical Pathologists has held yearly evaluation studies with the various author serologists acting as referees. These studies have tremendously improved syphilis serology by dramatically demonstrating the absolute necessity for the most strict adherence to the author's original technique. Also, the less sensitive and less specific techniques or technical variations have fallen by the wayside. With strict adherence to techniques, standard equipment and with availability of more satisfactory reagents and antigen, a level of uniformity is being approached by the majority of laboratories that is heartening indeed. And now with the advent of cardiolipin antigen and renewed interest in standardization, there is real hope for true uniform standardization of technical performance.

THE BIOLOGICAL FALSE POSITIVE OR FALSE POSITIVE SEROLOGIC TEST FOR SYPHILIS

Although the tests now is use have a high degree of specificity, a positive reaction does not categorically indicate syphilis. It only means that the test procedure used has detected a reagin or reacting substance of some type in the serum being tested. Since the lipoidal or cardiolipin test is empirical and non-specific, the substance giving the positive reaction may be due to syphilis or it may be due to a number of other conditions. Certain diseases are more prone to produce these reacting substances than others. Of particular interest and importance in the United States, now that the occurrence of a number of the infectious diseases which will be discussed has diminished, is the association of false positive reactions for syphilis with the so-called "collagen group" of diseases.

Collagen Disease

The problem of differentiation of the true positive from the false positive serologic test for syphilis has been in exceedingly complex and difficult one and has been complicated by the inability to make a definite diagnosis of presence or absence of syphilis in the latent stage when the only method available was the non-specific serologic test. With the

development of and increasing understanding of the significance of the TPI and related procedures which have a higher degree of specificity it has been found that a number of diseases such as disseminated lupus erythematosis, arthritis, etc., which have been collectively called diseases of the collagen system, are accompanied by serum changes which give rise to positive reactions in tests utilizing lipoidal antigens. At this time the relationship between the members of this group of diseases and the false positive reaction is not clear. There is, however, reason to believe that the false positive reaction has importance as an indicator of need for careful medical workup to determine the etiologic significance, ruling out syphilis in the process by use of one of the newer procedures such is the TPI test. This field has been most recently reviewed by Moore and Mohr, and it is anticipated that increasing availability of the TPI test or development of related tests using *T. pallidum* as antigen will eventually clarify this diagnostic problem.

Infectious Diseases

The false positive serologic test for syphilis has been reported to occur in association with a large number of infectious diseases, the more important of which will be discussed. It is important to remember that in yaws and pinta, both diseases caused by treponemata morphologically identical with the *T. pallidum*, the serologic response with both cardiolipin and lipoidal antigens as well as with the TPI or treponemal agglutination tests, is almost identical with that found in syphilis. In this group of so-called treponemal diseases, the diagnosis must be made on the basis of clinical and epidemiologic grounds, as no serologic test yet devised can make the distinction.

It has also been reported that false positive serologic tests are found in association with other venereal diseases, particularly in the acute phases of chancroid and lymphogranuloma venereum. The problem is a complex one, not yet well understood. It should be remembered that in the patient diagnosed as having a venereal disease other than syphilis on the basis of clinical and bacteriological findings, the burden of proof that the patient may not have a concurrent infection with syphilis rests with the clinician.

Malaria. Malaria is a prime offender, for apparently most cases of malaria may develop some transient false positive serologic tests for syphilis. About 10 to 20 per cent are significant enough to lead to diagnostic difficulties. The percentage varies remarkably in the different series of cases studied, partly because the time of taking the specimens

during the course of the disease, and the serological technique varied greatly. The highest percentage of false positive reactions occurs between 7th and 21st days of malarial seizures. There is no evidence of false positive serologic tests for syphilis in chronic malaria. Under treatment or after cessation of the malarial paroxysms the serum soon reverts to negative and any positive reaction persisting more than 4 to 6 weeks should be regarded with considerable suspicion. The spirochetal antigens and various verification tests have been of little help. However, the cardiolipin antigens only occasionally give false positive reactions in malaria.

Leprosy. Leprosy has long been known to cause persistent false positive serologic tests for syphilis. In the United States this has little significance because of the very low incidence of this disease.

Infectious Mononucleosis. Infectious mononucleosis frequently gives a transient and generally weak positive reaction for syphilis. Occasionally, a case will give a strong quantitative reaction and titers as high as 1:64 or higher are encountered. This reaction may appear relatively early in the disease; such false reactions usually appear during the 2nd week and disappear in about two weeks; they may persist up to 6 months. Although the rash and enlarged glands may cause some confusion with secondary syphilis, the quantitative titer in secondary syphilis is usually high. The spirochetal antigens, verification tests and cardiolipin antigens have not proven of specific help in this difficult situation.

Smallpox Vaccination. Smallpox vaccination in roughly 10 to 15 per cent of individuals results in transient weakly positive serologic tests for syphilis. Most of these cases became positive about 2 weeks after vaccination and a few remained positive when retested a week or two later. The reactions are generally weak of the doubtful type and quite transient. Typhoid and tetanus toxoid immunizations are not believed to produce false positive serologic tests for syphilis.

Atypical Pneumonia and Upper Respiratory Tract Infections. There exist large numbers of reports of false positive serologic tests for syphilis in cases of atypical pneumonia or virus pneumonitis. Careful study indicates that this probably does not occur in a significant number of such cases and furthermore that such reactions are usually weak, generally no more than doubtful and quite transient. The same can be said for upper respiratory infections. Furthermore, Davis has called attention to the fact that quite possibly some of these cases of upper respiratory tract infections are undiagnosed infectious mononucleosis.

Other Infectious Diseases. At one time or another most of the in-

fectious diseases have been reported as the cause of a false positive reaction. However, with technical improvements in performance of these tests and with increasing use of cardiolipin antigens there is no good evidence for believing that such infections as mumps, measles, whooping cough, diphtheria, and chicken pox, are frequent causes of positive serologic tests for syphilis.

Pregnancy, Menstruation, Malignancy

The literature contains instances of false positive reactions for syphilis in pregnancy, menstruation, and various forms of malignancy. Careful serologic studies in these conditions especially in recent evaluation surveys failed to confirm these reports. There is now no basis for believing that false positive serologic reactions for syphilis occur in these conditions.

THE VERIFICATION TESTS

With the realization that false positive serologic reactions for syphilis occurred in other diseases, serologists have attempted to distinguish true syphilitic reagin in a serum from other allied reacting substances. To this end a large number of procedures attempting to distinguish syphilitic from non-syphilitic serologic reactors have been devised. Wassermann's verification test failed, as have others since. The subject of the verification tests can be summarized by the statement that while a vast amount of work has been done on the subject, much of great theoretical interest, to date none of the procedures described by such workers as Kahn, Neurath, and others has proven to be of practical value for routine use nor have they proven to be sufficiently specific.

For routine serologic testing the use of cardiolipin in construction of antigens has very significantly improved the specificity of the procedure in which it is used. Thus the utilization of cardiolipin antigens has resulted in a significant increase of levels of specificity of the tests in common use. It can be said that the finding of a positive reaction by one of these procedures has such a high degree of probability of indication of the presence of active or past syphilitic infection that it is incumbent upon the clinician to rule out the presence of syphilis and then to determine the possible other cause of the reaction.

In this problem, the Treponema pallidum immobilization (TPI) test described by Nelson and Mayer represents a most valuable tool in distinguishing between patients with syphilis and suspected biological false positive reactors. Unfortunately, technical limitations of the procedure have precluded its widespread use.

In patients with untreated syphilis, the presence of treponemal immobilizing antibodies is regularly demonstrated after the primary or early secondary stage. Similar antibodies are formed in the other treponemal diseases, i.e., yaws, pinta, bejel, but so far as is now known such antibodies are not formed during the course of non-treponemal diseases. Thus, in the United States, a repeated positive treponemal immobilization test is almost certain indication of either past or present infection with syphilis. A negative TPI test may usually be assumed to exclude syphilis unless the patient has received antisyphilitic treatment early in the course of his infection or unless he is in the primary or early secondary stage in which the TPI test may be negative. In the infectious stages of syphilis the diagnosis can be and preferably should be made on the basis of the darkfield examination.

While it should be remembered that a single laboratory procedure should not by itself establish a diagnosis, a properly performed treponemal immobilization test interpreted in the light of the clinical situation can be one of the most valuable items of evidence in evaluating suspected biologic false positive reactors to serologic tests for syphilis utilizing lipoidal or cardiolipin antigens.

THE DIAGNOSTIC PROBLEM

Reagin in detectable amounts generally appears in the circulating blood soon after appearance of the primary lesion, the chancre, but it may require as long as a month after appearance for a test to become positive. Thus in the primary stage diagnosis is best made, and preferably made, by darkfield examination of serum expressed from the lesion. The finding of *T. pallidum* establishes the diagnosis. Treatment in the seronegative stage gives best results and is preferable both from the point of view of the patient and to stop at once the possible spread of the disease.

By the time the patient has entered the secondary stage, all of the lipoidal and cardiolipin tests are commonly positive. In this stage the diagnosis can be unequivocally established by the demonstration of *T. pallidum* in the lesions. If this cannot be done, the association of seropositivity with clinical findings and with history establishes the diagnosis with a high degree of certainty and provides ample justification for treatment.

The finding of a repeatedly positive serologic test for syphilis in the absence of obvious lesions or primary or secondary or late symptomatic syphilis calls for a most careful history and physical examination. The possibility of an unobserved primary lesion, intraurethral, cervical, or

extragenital, should not be overlooked. At the same time the physician must consider the possibility of asymptomatic central nervous system syphilis, of uncomplicated syphilitic aortitis, and of latency, for the diagnosis of syphilis is the responsibility of the clinical physician.

A positive serologic test for syphilis without any corroborative evidence from history, clinical examination, and spinal fluid examination, suggests one of the following conditions: (1) Latent syphilis. (2) A false positive reaction. (3) A laboratory technical error or a clerical error.

If a sample of serum is found positive, except in the instance of overt, darkfield positive primary or secondary syphilis, in which case the diagnosis is not dependent upon serologic testing, a repeat sample must be taken and examined. In this way it is possible to exclude laboratory or clerical errors.

If the sample is found repeatedly positive, further study is called for. The titer of the specimen is not too significant, for false positive reactions can be found to exist in high titer, while active asymptomatic central nervous system syphilis or latent syphilis can be found to exhibit very low titers. Thus spinal fluid examination and thorough workup are called for to rule out possible latent syphilis.

The false positive reaction must next be ruled out. The patient must be studied to rule out the presence of other diseases which are known to cause false positive reactions. In this, the TPI test will be found to be a valuable adjunct to other diagnostic procedures suggested by a consideration of the etiologic factors discussed in the preceding section. Repeated, titered, serologic testing is helpful in this study and should be done. The diagnosis of latent syphilis can be established by the finding of a positive TPI test. But in the absence of facilities for this procedure, the diagnosis can be established with a high degree of certainty by exclusion of possible causes of false positive reactions and by the finding of persistent seropositivity regardless of lack of history of lesions.

A positive serologic test for syphilis not accompanied by obvious signs of infectious or late symptomatic syphilis is not an emergency except in pregnancy. Rather, it calls for careful consideration with clear and deliberate thinking and for systematic workup of the patient to establish or disprove a diagnosis of syphilis, for in either event the diagnosis arrived at calls for medical management which is specific in the case of syphilis. If not syphilis, the determination of the etiology is most important to assure proper and intelligent management of other conditions which may be found.

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CHAPTER 20

CEREBROSPINAL FLUID

By Seward E. Miller

PHYSIOLOGY

The cerebrospinal fluid normally is a sparkling clear fluid, having the appearance of water, which fills the ventricles of the brain, the central canal of the spinal cord, and the mesothelial lined spaces between the pia and arachnoid membranes surrounding the brain and the cord.

FUNCTION

With the skull and vertebrae forming a rigid outer protective covering for the central nervous system, the spinal fluid acts as a fluid bed, protecting the brain and cord from jarring and trauma by softening and diffusing direct violence. The rigid bony protective covering also results in an inelastic fixed space for the brain, cord, and spinal fluid. Therefore, any change in volume of one of these structures is at the expense of one or more of the other structures. The elasticity of the meninges and of the blood vessels within this outer shell together with the freedom of communication between the ventriculo-subarachnoid spaces and the ample and freely anastomosing vascular channels allow shifts or dislocations of fluid and of blood from one part of the system to another. Also, this allows for compensatory volume changes between the different systems, enabling the cerebrospinal fluid to act as a regulating mechanism in maintaining a constant volume within the craniovertebral cavity. To some extent the fluid also plays a part in the metabolism of the tissues it bathes, acting as both a nutrient medium and as an excretory channel for the products of nervous tissue metabolism. The exact extent of its function in metabolism has not as yet clearly been established.

ORIGIN, FORMATION AND CIRCULATION

Normally the cerebrospinal fluid is produced by the choroid plexus in the ventricles with perhaps some slight additions from the ependymal cells of the ventricles and perivascular spaces of the spinal cord. Although the hydrostatic pressure within the highly vascular choroid plexus capillaries is believed to be greater than that of the spinal fluid, the

composition of spinal fluid differs materially from that of the blood plasma. Therefore, the process is not solely that of ultrafiltration through a selective permeable membrane but is much more complex. It probably involves a significant contribution on the part of the choroid plexus. Thus the cerebrospinal fluid represents more of a secretion of the choroid plexus and ependymal cells plus perhaps some selective reabsorption. True, the concentration of some of the constituents of the spinal fluid is directly influenced by their concentration in the blood plasma, so for practical purposes the cerebrospinal fluid is regarded as a dialysate in hydrostatic and osmotic equilibrium with the blood.

The fluid produced within the ventricles is believed to collect in the fourth ventricle, then escape from the fourth ventricle through the foramen of Magendie and the foramina of Luschka into the subarachnoid space, hence spreading over the brain and downward about the spinal cord. The normal daily production of spinal fluid has not been established but is estimated at between 100 and 150 ml., thus resulting in approximately a complete turnover of spinal fluid daily. In pathologic conditions it may be diminished or greatly increased. Since the pressure of the cerebrospinal fluid is greater than that within the veins and sinuses absorption takes place, for the most part, from the subarachnoid space into the meningeal veins of the cerebral hemispheres and relatively little through the membranes of the vertebral canal. Evidence points to the major absorption by way of the arachnoid villi into the dural sinusoids, but there is also convincing evidence for some absorption by way of the meningeal capillaries and the lymphatics of the perineural spaces.

The total volume of cerebrospinal fluid varies greatly with the size of the individual but normally is between 100 and 150 ml. in adults. Also under normal conditions its rate of formation, composition, and absorption within rather narrow limits is quite constant. In the presence of injury or disease permeability of the membranes is altered to varying degrees with the appearance of inflammatory reaction resulting in interference with the circulation of the fluid. Careful examination of the spinal fluid frequently is of great significance, enabling the physician to detect abnormalities, estimate the degree of involvement and in some diseases demonstrate or establish a specific etiologic agent. Correlation of the abnormalities in the spinal fluid with the clinical findings of the physician frequently results in most significant aid in diagnosis, prognosis and treatment.

REMOVAL OF CEREBROSPINAL FLUID

Lumbar Puncture

Indications and Contraindications. Examination of the cerebrospinal fluid is indicated whenever there is clinical evidence of central nervous system disease, in some cases of injury, and following treatment of early syphilis or in asymptomatic tertiary syphilis. Removal of spinal fluid also is indicated frequently for relief of abnormal pressure and for the drainage of exudates or blood. In addition there are two further indications for lumbar puncture and removal of spinal fluid: for diagnostic purposes in visualization of the central nervous system by the injection of air or lipoidol, and lastly in treatment, for the injection of drugs, serums or for spinal anesthesia.

Although lumbar puncture is a relatively safe and simple procedure, it should not be done unless there are definite indications, with the possible advantages to the patient outweighing the disadvantages, and unless the operator is familiar with the anatomy of the region and trained in surgical technique. Although cisterna puncture is technically simple it is not recommended except in case of block in the spinal canal. The major contraindications are subtentorial tumors with the presence of greatly increased intracranial pressure as manifested by clinical symptoms, papilledema and roentgenographic skull changes. Punctures should not be done through infected areas or in the presence of obvious spinal deformity. The procedure is ill advised as a rule in very elderly people, in severe cachectic states, in circulatory failure or in neurotic individuals without evidence of organic lesions of the nervous system. Opinion is divided as to the indications and value of routine lumbar puncture in head injuries. It should be emphasized that signs and symptoms of increased intercranial pressure, or the suspicion of brain tumor, abscess or hemorrhage, are indications for a diagnostic spinal puncture rather than counterindications. However, in all such instances the fluid should be removed slowly and generally under manometric control.

Excellent descriptions of the preparation of the patient, instruments, and technique of spinal and cisternal puncture abound in many texts of neurology and surgery and therefore will not be repeated here.

Collection of Specimens. If the fluid is under greatly increased pressure it should be removed slowly. If the fluid is cloudy (purulent meningitis) 20 to 30 ml. can be safely removed, but if the fluid is clear and under increased pressure as with an expanding mass, the abnormal pressure should not be reduced more than half.

For routine tests, 3 to 4 ml. of fluid are collected in each of three sterile test tubes held beneath the needle hub. The tubes should be *numbered in the order of collection.* After the collection the manometric reading again should be taken.

Cisterna Puncture

Indications and Contraindications. Cisterna puncture is indicated when the diagnosis or treatment is dependent on the entry into the subarachnoid space for the purpose of removal of fluid or the injection of therapeutic or diagnostic agents and when lumbar puncture is impossible, contraindicated, or will not suffice. Cisterna puncture is sometimes used in conjunction with lumbar or ventricular puncture to aid in the localization of lesions or for irrigations. The major contraindications are subtentorial tumors or adhesions at the base of the brain.

Cisterna puncture in the hands of experienced operators familiar with the anatomy of the suboccipital region and the hydrodynamics of the cerebrospinal fluid is relatively simple and safe, but the procedure is not to be performed by the occasional operator.

EXAMINATION OF CEREBROSPINAL FLUID

General

The minimal examination on cerebrospinal fluid should include the following procedures:

1. Pressure (manometric). Response to jugular compression.
2. Physical examination (color, turbidity, pellicle, sediment).
3. Cytologic examination (cell count).
4. Chemical examination (quantitative protein).
5. Serology.

In the presence of meningeal signs, cloudy fluid or increase in cell count, additional examinations are indicated:

1. Differential cell count.
2. Smears for bacteria (Gram's, Ziehl-Neelsen stain).
3. Culture.
4. Quantitative sugar.
5. Colloidal tests.

In selected cases additional examinations are indicated—quantitative chlorides, calcium, urea, bromides, alcohol and, if tuberculosis infection is suspected, the Levinson and tryptophane tests, and animal inoculation.

Dynamics

Manometers. Cerebrospinal fluid under normal pressure flows from the needle at the rate of 20 to 60 drops per minute, but the estimation of pressure by noting the speed of flow from the needle is grossly unreliable, for it does not take into account such variable factors as size and patency of needle, partial occlusion of top, viscosity of fluid or temporary physiological alterations in intradural pressure. True evaluation of the pressure is best obtained by the use of manometers. The manometers most used are the straight tube water manometer and the U-tube mercury type.

Interpretation. With the patient in the horizontal position the pressures in the ventricles, cisterna magna and subarachnoid spaces are the same. The normal adult pressure in the horizontal position is 125 mm. water, with a range of 75 to 200 mm., or up to 8 mm. of mercury. Before slight elevations above 200 mm. can be interpreted as significant one must rule out physiological elevations due to fear, cramped position, elevation the head or breath holding. Pressures above 250 mm. are probably pathologic.

When the patient is in the upright position the pressure of the spinal fluid in the lumbar region is increased. Therefore, manometric readings should always be taken with the patient in the recumbent position.

The pressure of the cerebrospinal fluid depends on the balance between the rate of formation and the rate of absorption and upon the reciprocal relations between the fluid volume and the volume of the blood vessels, brain substance and other structures within the fixed cranio-vertebral bony framework. Factors influencing the pressure are the hydrostatic pressure within the capillaries and veins, the osmotic pressure of the blood, the rate of expansion of lesions, the freedom of communication of subarachnoid spaces and the condition of the meninges and blood vessels. The fluid pressure is not significantly altered by variations in the arterial blood pressures.

Low manometric readings are often observed in normal individuals, hence a reduced pressure is of little diagnostic significance. However, the pressure is low in such conditions as fainting, shock, dehydration, diabetic coma, subarachnoid block and chronic degenerative lesions. Following the intravenous injection of hypertonic fluids such as glucose, sucrose, magnesium sulphate and sodium chloride, water is withdrawn from the subarachnoid spaces and the pressure is temporarily reduced.

The most common cause of increase in fluid pressure is inflammation

of the meninges. Inflammatory lesions alter the permeability of the membranes. There is an increase in hydrostatic pressure within the vascular bed, interference with absorption and sometimes a lowered osmotic pressure of the blood. The degree of pressure increase varies with the location, extent and severity of the lesion. In low grade inflammatory processes as in poliomyelitis, encephalitis, benign lymphocytic meningitis and neurosyphilis the pressure is usually moderately increased (250 to 500 mm.). In tuberculous meningitis and in acute purulent meningitis the pressure may be increased above 500 mm. Pressures greater than 1,000 mm. are seldom observed, for pressure above this level causes a collapse of the capillaries and veins.

Other conditions which cause elevation of cerebrospinal fluid pressure are expanding lesions such as brain tumor, abscess, aneurism and hematoma, edema of the brain, cerebral and subarachnoid hemorrhage, hypotonic intravenous medications, irritant substances injected intrathecally and numerous drugs. In acute febrile diseases the osmotic pressure of the blood is decreased, causing an increase in the rate of cerebrospinal fluid formation and elevation in fluid pressure (meningism). Conditions in which there is venous stasis, as in congestive heart failure, respiratory embarrassment, mediastinal and cervical masses interfering with venous return to the heart, thrombosis of venous sinuses and meningeal veins, result in an increase in spinal fluid pressure.

Compression of the jugular veins produces stasis in the intracranial veins, causing them to become distended. The resulting increase in vascular volume causes an immediate increase in cerebrospinal fluid pressure. Release of the jugular compression restores the normal venous pressure and the spinal fluid pressure promptly returns to normal. Firm compression of the jugular veins results in a rise of 150 to 300 mm. Failure of the fluid to rise on jugular compression and to fall on release of pressure indicates subarachnoid blockage, whereas a delayed rise or fall indicates a partial block. In case of lateral sinus or jugular thrombosis, pressure on the jugular vein on the affected side causes no spinal fluid pressure change; however, there is a normal response on the opposite side (Tobey-Ayer test). Pressure alterations of unequal intensity in two puncture sites observed simultaneously may be of aid in localization of lesions.

Removal of fluid decreases the pressure. The amount of drop in pressure with fluid removal varies widely, the decrease being of the order of 5 to 10 mm. for each ml. removed. By noting the pressure before and after the collection of fluid a gross idea may be obtained as to the vol-

ume of spinal fluid. A slight fall (10 to 20 mm.) after removing 10 to 20 ml. indicates a large volume as in hydrocephalus, whereas a marked fall points to a small reservoir as in space-occupying mass or spinal block.

Gross Examination

Normal cerebrospinal fluid is a thin, sparkling, colorless fluid with no sediment; it does not coagulate. It has an alkaline reaction with a pH of 7.4 to 7.5 and a specific gravity of 1.003 to 1.008.

Turbidity. Turbidity may be caused by blood, inflammatory cells or bacteria. In order to detect slight degrees of opacity it is necessary to hold the tube in a good light against a dark background. A clear fluid does not rule out significant pleocytosis or early meningitis. Clear fluids exist in central nervous system syphilis, meningismus, diabetic coma, lead poisoning, and even in poliomyelitis and tuberculous meningitis. However, a frankly purulent fluid means meningitis. A visible haziness is produced when the cell count is 500 cells or more per cubic millimeter. Fluids with cell counts less than 500 appear clear.

Clot. In conditions in which there is injury to the meninges of a degree sufficient to cause an increase in proteins including fibrinogen, the fluid clots. In sacculated lesions in which the protein content is greatly increased the fluid may undergo coagulation within a few minutes after it is removed. With less fibrinogen concentration minor coagulation or the formation of a fine web or pellicle does not occur until hours after removal. Clot formation is the rule in purulent and tuberculous meningitis, whereas there is less likely to be pellicle formation in poliomyelitis or encephalitis.

Blood. A frankly bloody fluid due to faulty technique usually results in blood only in the first tube collected. Blood in the fluid as a result of vascular pathology is likely to be uniform in all tubes, does not coagulate, and on standing on centrifugalization leaves a yellowish supernatant fluid from frank hemorrhage into the ventriculo-subarachnoid spaces or as the result of diapedesis of red cells from areas of stasis or from hemorrhagic extrameningeal lesions.

Xanthochromia (Yellow Fluid). Yellow discoloration of the fluid may be due to blood pigments resulting from red cell disintegration with the subarachnoid space or to altered permeability of membranes to pigments which under normal conditions are excluded. Conditions in which xanthochromia may be found include subarachnoid hemorrhage, extradural and sub-pial hematoma, spinal block, tumor, abscess and acute

inflammation. Xanthochromia is apparent a few days after hemorrhage, deepens for several days, and if there are no more hemorrhages slowly disappears (1 to 2 weeks). Yellow spinal fluid may give a positive reaction to the benzidine test, to the van den Bergh test or to neither, thus indicating lack of uniformity in its causation. In the usual case of jaundice the spinal fluid is not discolored, but in severe and chronic jaundice, xanthochromia may be present. Usually when there is xanthochromia there is also an increase in protein and in cells, but there is no direct correlation in the degrees of increase of these elements. A marked xanthochromia with a greatly increased protein content represents Froin's syndrome which occurs below a lesion blocking the spinal canal.

Cell Count

General. Cell counts on cerebrospinal fluid should be made as soon as possible after collection—best within one half hour, for the cells disintegrate on standing and uniform cell suspensions are difficult to obtain after the cells have settled or fibrin has formed.

The normal cell count ranges from 0 to 8 and these cells are small lymphocytes with round or oval nuclei. Rarely large lymphocytes are found while polymorphonuclear leukocytes are found only in pathological fluids. The cell count should be made from the third tube of fluid for this tube is less likely to be contaminated by blood originating as a result of the puncture. Although methods have been devised for estimating a low pleocytosis in bloody spinal fluid most workers place little reliance in them so a white cell count on bloody fluid is of little value and generally not attempted. Red-cell counts frequently are done on bloody fluids to serve as a basis for comparison with future fluids to determine increase or decrease of hemorrhage.

Method. The use of a white blood cell counting pipette, standard hemocytometer and a glacial acetic acid crystal violet diluting fluid are recommended. The diluting fluid is drawn up to mark 1 in the leukocyte pipette and the spinai fluid up to mark 11. After thorough mixing the first 2 or 3 drops are discarded, then a drop is placed on each side of a double counting chamber as for a leukocyte count and the preparation allowed to stand for 2 to 3 minutes for the cells to settle. Red blood cells will be hemolyzed and the nuclei of the white blood cells stained so that errors of confusion with artefacts are greatly reduced. Count all the cells within the entire ruled areas on both sides of the counting chamber. Since there are 9 1-mm. squares on each side, the total count

obtained in counting the 18 squares is multiplied by 0.6 to obtain the number of cells per cubic milliliter of undiluted fluid.

Interpretation of the White Cell Count. The most frequent errors in the white cell counting methods are delay in counting, failure to mix fluid thoroughly, and confusion of leukocytes with artifacts or erythrocytes. Great accuracy is more necessary in fluids with slight pleocytosis than in fluids with high counts. The normal number of white blood cells is 0 to 8 per cubic milliliter. Counts above 10 are indicative of pathologic change. In the presence of localized, chronic or degenerative lesions in which there is no or slight inflammatory reaction in the meninges or adjacent to them, as in meningismus, brain tumor or multiple sclerosis, the cell count may be normal or only slightly elevated. In lesions characterized by cellular exudation of a moderate degree, as in poliomyelitis, encephalitis, or neurosyphilis, there is a moderate increase in cells (10 to 200). In tuberculous meningitis the cell count is usually only moderately increased. The highest counts (1,000 to 10,000) are found in acute suppurative meningitis. There is great variability in the cell count in different individuals with the same disease and in the same individual at different stages of the disease process, so that the cell count alone cannot be considered as having diagnostic specificity. Nevertheless pleocytosis is a most significant barometer of a central nervous system lesion.

Differential Count

Method. When the fluid is clear, or only slightly cloudy, centrifugalize and examine the sediment. When the fluid is definitely cloudy centrifugalization is not necessary. Differential counting methods on spinal fluid are relatively unsatisfactory, for the cells are poorly preserved and good differential staining is difficult. Numerous cell types are found in cerebrospinal fluid, but for practical purposes the separation of cells into neutrophiles, lymphocytes and large mononuclears is sufficient.

The smear may be dried, stained and examined just as a blood smear for a differential count, or a most satisfactory and simple method of doing a differential count is to add methylene blue (Löffler's alkaline) to the fluid and make a moist preparation of the mixture. The methylene blue may be added to the sediment (3 drops per ml.) or a small drop of methylene blue added to a medium-sized drop of fluid on a slide. A coverslip is placed on the drop and it takes 5 to 15 minutes for the best staining effect. The advantages of the moist preparation over the dried

smear are that the cells are more concentrated, there are fewer artefacts, there is less cellular distortion and better cell definition.

Interpretation. In the fluid from normal individuals the cells are practically all small lymphocytes with an occasional cell of the large mononuclear type. In chronic and low grade infections with moderate pleocytosis, and in tuberculous and syphilitic meningitis, the predominating cells are lymphocytes. In acute inflammatory conditions, whatever the cause, but particularly in diseases caused by pyogenic organisms as meningococci and pneumococci, the cells of the neutrophilic granulocyte series are predominant. In localized pyogenic infection as cerebral or extradural abscess, in hemorrhage and in response to substances injected into the subarachnoid space there is usually a neutrophilic response. A rising neutrophilic percentage is a sign of a progressive lesion, whereas an increase in the lymphocytes on successive examinations is a favorable prognostic sign.

Bacteriologic Examination

The tubercle bacillus (*Mycobacterium tuberculosis*), meningococcus (*Neisseria intracellularis*), pneumococcus (*Diplococcus pneumoniae*) streptococcus, staphylococcus and influenza bacillus (*Hemophilus influenzae*) are the organisms most frequently demonstrated in the cerebrospinal fluid, but numerous other bacteria are occasionally found. In meningitis due to yeast or to fungi and in certain parasitic diseases as trypanosomiasis and trichiniasis the etiologic agent is demonstrable.

Gram's Stain. Gram's stain is indicated in the presence of meningeal signs, cloudy fluid or pleocytosis. If the fluid is clear or slightly cloudy, smears are made of the centrifugalized sediment. If the fluid is frankly purulent, smears are made without preliminary centrifugalization. After drying and fixation with gentle heat the Gram's stain is applied.

Meningococci are Gram-negative diplococci, varying in size, and are both intra- and extracellular. Usually the organisms are few in number and careful search is necessary. When neutrophils are numerous but there are no demonstrable organisms, meningococcic meningitis is the most likely possibility. In the other coccal infections, the organisms are generally numerous. The Gram-positive organisms often stain atypically and may be mistaken for meningococci, hence it is well to wait for cultural confirmation before making the final diagnosis The contamination of specimens by non-pathogenic organisms frequently occurs, and for this reason it is necessary to observe strict bacteriologic technic and to examine specimens promptly. In the

presence of more than one type of organism, or many organisms in the absence of reactive cells, contamination may be assumed. Artifacts and precipitated stain may simulate organisms closely, but an experienced microscopist usually can differentiate these from bacteria.

Ziehl-Neelsen Stain for Tubercle Bacillus. In clear or slightly turbid fluids from patients with meningitic signs in which there are no demonstrable organisms in the Gram's stain and a lymphocytic pleocytosis, search should be made for tubercle bacilli. The tubercle bacilli are likely to be entrapped in the fibrin web, hence smears made from the pellicle are most likely to yield positive results. By examining both pellicle and centrifugalized sediment the chance of demonstrating the organisms is increased. After drying, the Ziehl-Neelsen (acid fast) stain is applied. Diligent search is required to demonstrate the tubercle bacillus. The failure to find the organism does not rule out tuberculous meningitis.

Culture. Cultures of cerebrospinal fluid are indicated in the presence of meningeal signs and as a confirmatory and differential diagnostic procedure when organisms are demonstrated in the smears. The most favorable method of inoculation is to allow 1 to 2 ml. or more of fluid to flow directly from the lumbar puncture needle into warm culture medium, which is promptly transferred to the incubator. Satisfactory results, however, may be obtained if the fluid is sent to the bacteriological laboratory for inoculation. Chilling is to be avoided and refrigeration is contraindicated. It is preferable to pipette several ml. of fluid into the culture medium rather than to transfer a loop full. Centrifugalization is unnecessary. Plate cultures are prepared by allowing 1 to 2 ml. of spinal fluid from a sterile pipette to flow across the surface of the plated medium.

Cultures for tubercle bacilli using a sensitive medium are positive in a large percentage of cases of tuberculous meningitis. See Chapter 10, Medical Bacteriology, for discussion of culture media.

Animal Inoculation. For the confirmation of the diagnosis of tuberculosis, 1 to 2 ml. of sediment of cerebrospinal fluid is injected beneath the loose skin of the groin of a guinea pig. The animal is killed after 6 weeks and the diagnosis made by the demonstration of the tubercle bacillus in the caseous material from the lymph nodes or spleen.

Chemical Examination

No laboratory examination is complete without an adequate chemical examination. The types of chemical examinations indicated vary with the clinical symptoms and type of fluid.

Protein. The qualitative tests are so grossly inaccurate that rapidly they are being replaced by accurate quantitative determinations. A rough qualitative test may be simply performed by placing 1 ml. of saturated ammonium sulfate in a test tube and overlaying it carefully with an equal volume of spinal fluid (Ross-Jones test). A white ring at the layer of contact indicates increased protein in the spinal fluid. No precipitate forms with normal spinal fluid. A slightly more sensitive test (Pandy's) is performed by adding one drop of spinal fluid to 1 ml. of saturated aqueous phenol solution in a test tube. Normal spinal fluids give a very faint turbidity while fluids with increased protein

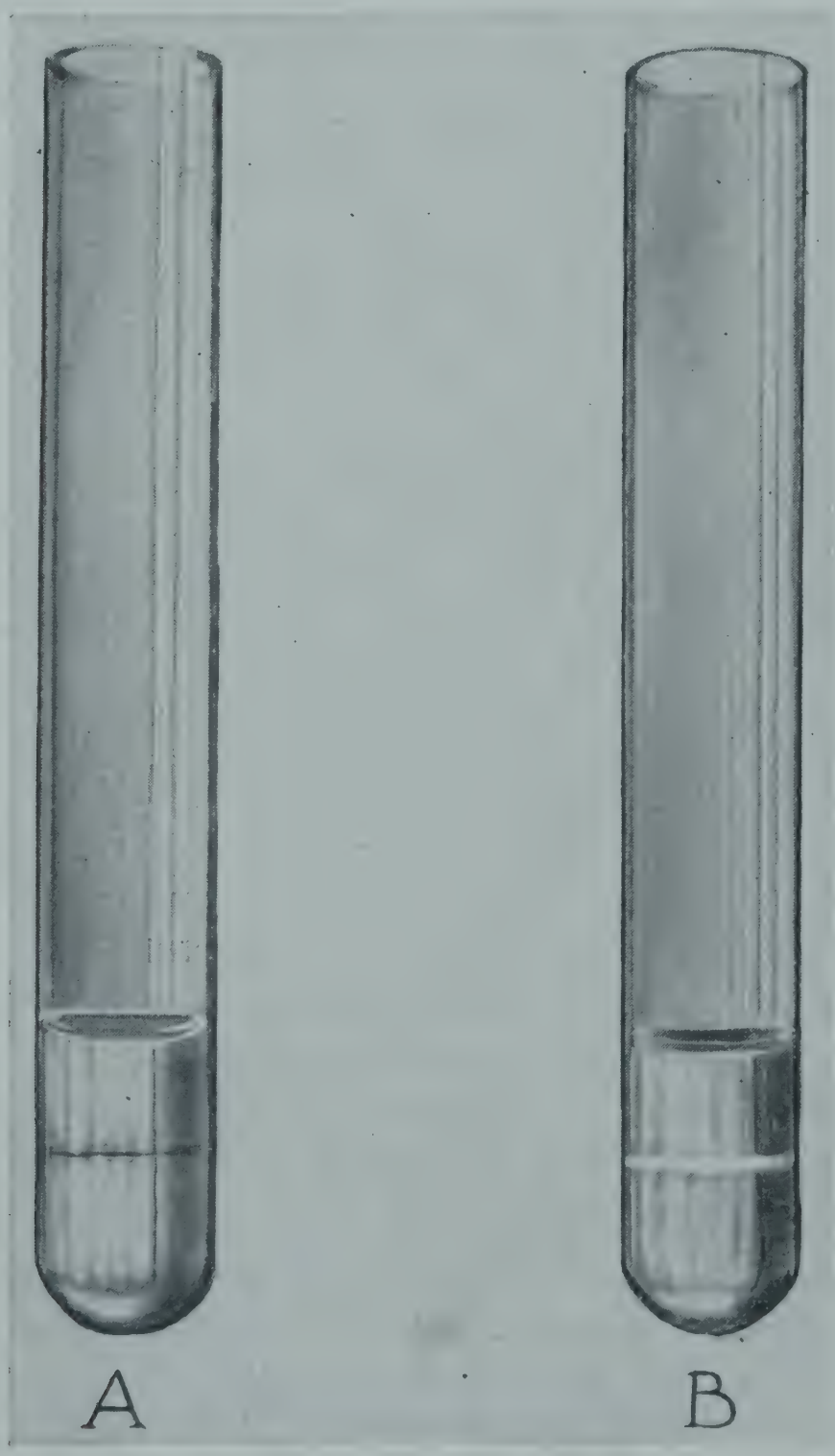


FIG. 20.1. Test for globulin in spinal fluid. A. Negative test. B. Positive test showing cloudy ring at junction of ammonium sulphate solution in bottom of tube and spinal fluid above.

produce a definite white cloud. This test is useful particularly when the amount of spinal fluid available is small.

Accurate Colorimetric Total Protein Determinations

One of the accurate and rather simple chemical methods for determining quantitatively the amount of protein present in spinal fluid should be used routinely. The simplest and quite accurate for all clinical purposes is the turbidity type test utilizing sulphosalicylic acid to precipitate the protein. Such tests are inexpensive and give the clinician a report in specific amounts (mg.) of protein present. So important is this greater accuracy, particularly in the diagnosis of a ruptured inter-

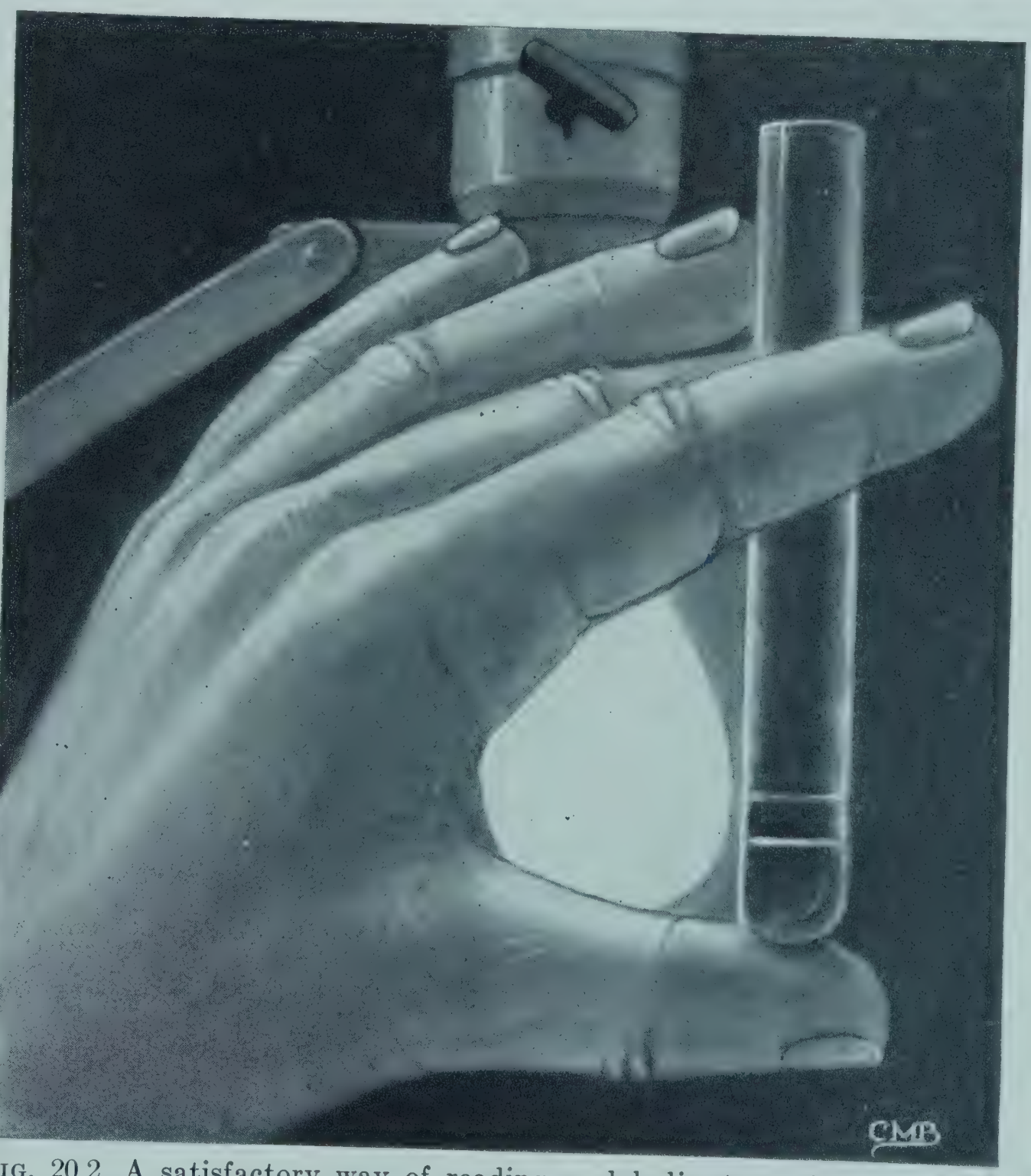


FIG. 20.2. A satisfactory way of reading a globulin test. The tube is held before a black background with light slightly from the side.

vertebral disk and early lesion of the central nervous system, that the routine use only of the Pandy and other qualitative methods should be discarded in favor of this type of quantitative measurement. If a photoelectric colorimeter is not available, the test can be performed with an ordinary visual colorimeter using a modified Dennis-Ayer method.

Discussion. Blood and contamination by bacteria or fungi cause high protein readings If the fluid is sterile and kept in the refrigerator the protein content is not appreciably changed for several weeks.

If small colorimeters or special attachments for standard colorimeters are available, the quantitative protein test can be performed with small quantities of cerebrospinal fluid.

Interpretation. When the fluid at the time of removal is cloudy or grossly bloody or when a clot (fibrin) is demonstrable, the protein is increased and qualitative chemical tests are superfluous. Tests for protein should be routine on all clear fluids, for often an increase in protein may be the only demonstrable abnormality.

The qualitative tests are not satisfactory for they do not measure the degree of protein increase. The quantitative test is needed, particularly in diseases in which a slight increase is of considerable diagnostic value as in tumors, rupture of an intervertebral disc and in low grade inflammatory lesions.

The protein in normal cerebrospinal fluid varies from 15 to 40 mg. per 100 ml. with an average of 25 mg. In the presence of disease of the central nervous system the permeability of the membranes to serum proteins is altered, and the total protein is increased. The highest protein concentrations (200 to 1000 mg.) are found in acute meningitis, subarachnoid hemorrhage or in sacculation of fluid. The combination of a high protein content, xanthochromia and generally lymphocytic pleocytosis is known as *Froin's* syndrome and is most frequently present in conditions causing spinal subarachnoid blockage. In practically all organic neurologic diseases there is a significant rise in protein content. The degree of increase is a measure of the severity of the lesion.

In normal fluids albumin is the predominant protein, but in disease the globulin fraction is relatively increased and fibrinogen and other proteins appear. The quantitative differentiation of these various protein fractions has little practical value.

Sugar

Qualitative tests for sugar are unreliable and yield so little information that their use on spinal fluid is not recommended. The quantitative

method most widely used is that of Folin-Wu. Spinal fluid diluted 1:5 is generally used instead of the usual filtrate. Protein free filtrate must be prepared if the protein content is elevated.

Interpretation. Normal spinal fluid sugar varies from 50 to 80 mg. per 100 ml. The values obtained by reduction methods are about one-half of the values obtained on blood removed at the same time. In the presence of sustained increases or decreases in blood sugar concentration there are alterations in the cerebrospinal fluid values in the same direction, hence the high values in diabetes or following intravenous glucose and the low values in hyperinsulinism and starvation. Lack of correlation between blood and spinal fluid sugars may be due to slowness of diffusion, to greater relative quantity of reducing substances other than sugar in the blood and to glycolysis. The quantitative glucose test is more informative if the fluid is collected under fasting conditions and a specimen of blood is analyzed for sugar content at the same time. Glycolysis occurs rapidly on standing at room temperature especially in the presence of bacteria, hence the necessity of prompt examination or of preservation (crystal of thymol).

The cerebrospinal fluid sugar is normal or slightly increased in conditions in which there is increase in intracranial pressure in the absence of marked pleocytosis as in encephalitis, brain tumor and neurosyphilis.

The chief indication for quantitative sugar determinations is the suspicion of meningitis. The sugar is markedly reduced in bacterial infections of the meninges, the values being below 50 mg. as a rule and often below 25 mg. A normal or high sugar content in the presence of pleocytosis is indicative of a non-bacterial inflammation. Sugar determinations are of value in differentiating localized inflammations as brain abscess or extradural lesions (sinus or mastoid) from diffuse meningitis, for the localized lesions give normal values. Sugar determinations may also be of value in establishing the prognosis of meningitis and in directing therapy, for a falling sugar is the sign of progress of the lesion and a stationary or rising sugar is a sign of improvement.

Low spinal fluid sugar levels are found in cases with diffuse leptomeningeal tumor metastasis, although normal sugar values are present in cases with solitary or scattered nodular metastatic lesions. This has been explained by the sheets of tumor cells utilizing sugar from the spinal fluid directly, in their metabolism.

In unexplained coma the tests for sugar aid in establishing or ruling out the diagnosis of diabetes.

Chlorides

Determine as for blood, using 1 ml. of spinal fluid and 9 ml. of water (see Chapter 7, Blood Chemistry).

Interpretation. The concentration of the sodium chloride in normal cerebrospinal fluid ranges from 720 to 750 mg. per 100 ml. The concentration of the plasma sodium chloride is 550 to 620 mg. per 100 ml. The concentration of fluid chlorides varies as the chloride content of the serum. The chlorides are increased when there is hyperchloremia, as in kidney disease with nitrogen and salt retention. Thus in cases of coma or of unexplained convulsions the estimation of chlorides in the cerebrospinal fluid may direct attention to the possibility of uremia. The chlorides are decreased in any condition in which there is hypochloremia as in acute infections, vomiting, diarrhea or profuse sweating in the presence of adequate water intake.

The chief use of chloride determinations is in the differential diagnosis of meningitis and in prognosis. In acute meningitis the chloride content is decreased, usually below 600 mg. The decrease is due to a lowered chloride concentration of the blood, to alteration of permeability of the meninges and to increased proteins in the cerebrospinal fluid. In localized inflammations, in non-bacterial inflammations with pleocytosis, in brain tumor and in hemorrhagic lesions the chlorides are as a rule normal. In tuberculous meningitis the sodium chloride concentration is usually less than 650 mg. and often less than 550 mg. per 100 cc., whereas in encephalitis, poliomyelitis, benign lymphocytic meningitis and neurosyphilis, conditions which often present clinical syndromes resembling tuberculosis, the chlorides are normal. The finding of a clear fluid under increased pressure with pleocytosis, increased proteins and with low sugar and chlorides constitutes a combination of findings which is practically diagnostic of tuberculous meningitis.

Urea

The concentration of urea in the fluid is approximately the same as that of the blood (12–15 mg. urea N per 100 ml.). In the presence of kidney failure the urea nitrogen may be increased to twice or more the normal value. Actually all the non-protein nitrogenous elements parallel the amounts of these substances in the blood and therefore such examinations are seldom performed except in the absence of a satisfactory blood specimen.

Calcium

The calcium of the normal cerebrospinal fluid varies from 4 to 6 mg. per 100 ml., which is approximately one-half that of the blood. The calcium appearing in the fluid is that fraction not bound to proteins, that is, diffusible calcium. In recent years numerous investigations of the spinal fluid calcium have been made in order to determine its significance in convulsive states, toxemias and other conditions, but the result so far has been disappointing. Calcium is increased in the spinal fluid in conditions in which the spinal fluid protein is increased.

Acid-Base Equilibrium

The pH of normal spinal fluid is between 7.4 and 7.6 with a CO_2-combining power of 50 to 60 volumes per cent. The pH remains normal in meningismus, most cases of central nervous system syphilis, most tumors, hydrocephalus, and serous meningitis. The pH is lowered (acidosis) in inflammatory lesions of the meninges, especially acute meningitis.

Lead, Alcohol, Bromides

These and other toxic substances may be found in spinal fluid. The chemical examination is performed as with blood. Demonstration of a toxic substance is a finding of great clinical significance.

Levinson and Tryptophane Tests for Tuberculous Meningitis

Levinson Test. Method. One ml. of cerebrospinal fluid to be tested is placed in each of two serological tubes of equal size (about 6 mm. in diameter). One ml. of 2 per cent aqueous mercuric chloride solution is added to the first tube, and 1 ml. of 3 per cent aqueous sulphosalicylic acid is added to the second. The tubes are stoppered, the solutions mixed, and allowed to stand in an upright position at room temperature for 24 hours. The height of the sediment in the two tubes is measured and compared.

Interpretation. In normal cerebrospinal fluid the sediment in both tubes is less than 2 mm., but in fluids in which there is an increase in proteins the sediment in both tubes is heavy. The sediment in the mercuric chloride tube is light and feathery and forms slowly. The sediment in the sulphosalicylic acid tube is compact and heavy and forms rapidly.

In tuberculous meningitis the height of the sediment in the mercuric chloride tube is 2 mm. or more, and is two or more times that in the sulphosalicylic acid tube. In suppurative meningitis the sediment in the sulphosalicylic acid tube is greater than in the mercuric chloride tube.

The Levinson test finds its greatest usefulness in the diagnosis of tuberculous meningitis. In this disease the 2 to 1 ratio of the mercuric chloride to sulphosalicylic acid is found in more than 90 per cent of cases. The same sort of reaction, however, is found in a wide variety of other conditions, so that the test is not specific for tuberculosis. Other disadvantages of the method include uneven settling of precipitates making accurate measurement difficult, and border-line ratios which are difficult to interpret.

Tryptophane Test. Method. Two to three ml. of fluid are placed in a medium sized test tube, and 15 ml. of concentrated hydrochloric acid and 2 to 3 drops of 2 per cent formaldehyde added. The mixture is shaken and allowed to stand for 5 minutes. Then several ml. of 0.06 per cent aqueous sodium nitrite is carefully overlaid. A negative reaction is characterized by an absence of color change at the junction of the two fluids or by a faint yellowish color. A positive reaction is characterized by a delicate violet ring which may take several minutes to become visible and which persists for fifteen minutes or longer.

Interpretation. Positive tryptophane tests are obtained in grossly bloody fluids, in purulent and in xanthochromic fluids, whatever the cause, hence the test has no value in fluids of this character. Fluids containing less than 400 red blood cells per cu. mm. do not give false positive reactions. The test is of value in the diagnosis of diseases with clear cerebrospinal fluid, for the purple ring is demonstrable in about 95 per cent of the cases of tuberculous meningitis, whereas in encephalitis, poliomyelitis, brain tumor and in meningitis due to syphilis the reaction is usually negative. The delicacy of the ring and the number of false positive reactions tend to discredit this test as a substitute for other methods, but its use as a corroborative procedure in the differential diagnosis of tuberculous meningitis is well worth while.

Serologic Tests for Syphilis

Involvement of the central nervous system occurs in about 25 per cent of patients infected with the Treponema pallidum. Serologic tests for syphilis made on the cerebrospinal fluid are the most specific of all tests for the detection of central nervous system involvement but give no indication as to the type of neurosyphilis. The Wassermann

or other complement fixation tests are considered by most authorities as the most dependable of the serologic tests for use with cerebrospinal fluid. (See Chapter 19, Syphilis Serology.)

A negative serological test on cerebrospinal fluid is presumptive evidence of lack of syphilitic involvement. Doubtful reactions (the so-called 1 and 2 plus) should not be considered diagnostic, unless there is supporting evidence. The provocative test after intravenous arsphenamine is of no more value than a second test would be without arsphenamine. A definitely positive serologic test, even in the absence of other abnormalities may be considered as proof of neurosyphilis until proved otherwise. The cerebrospinal tests for syphilis may be positive when the blood tests are negative. Positive tests may appear years before there is clinical evidence of nerve tissue involvement. The serologic tests are of value in planning treatment and in prognosis. Failure of serologic response to specific thereapy is indicative of diffuse parenchymatous involvement with poor prognosis.

Colloidal Tests

General. When normal cerebrospinal fluid in varying saline dilutions is mixed with colloidal suspensions, there is no alteration in the colloidal state. When the fluid is abnormal there is color change or precipitation of colloidal particles. The changes taking place do not occur uniformly in all dilutions but are maximum in certain ranges of dilution, as in the first, middle or end zones. The explanation for the reaction is not known, but it is associated with a protein increase and alterations in the ratio of the protein fractions, probably in relation to the increase of gamma globulin.

The first colloidal test introduced was the colloidal gold test (Lange, 1912), which was thought to be specific for neurosyphilis and for the differentiation of the types of syphilitic lesions. The test was universally adopted and is still used in many laboratories the world over, but its specificity and utility as a diagnostic and differential diagnostic procedure has been shown to be much less than was at first claimed. The colloidal gold solution is expensive, requires careful standardization, and is technically difficult to prepare. Because of these objections, numerous other colloidal tests and modifications have been developed, of which the gum mastic, colloidal benzoin and paraffin tests are the most widely used. Numerous and thorough comparative studies have been made and the consensus of opinion is that the more simple tests are equally as sensitive, are as informative as the colloidal gold test

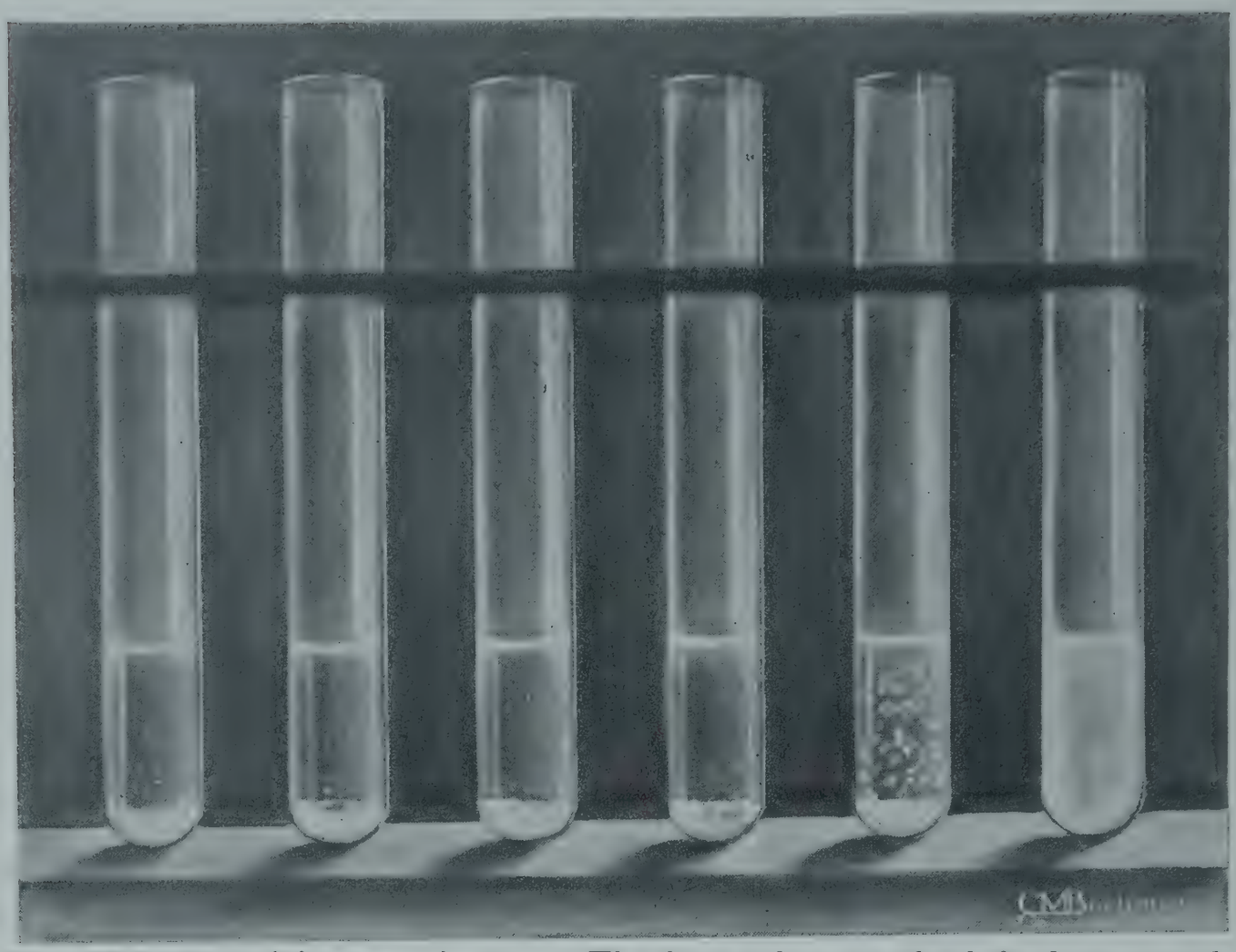


FIG. 20.3. A positive mastic test. The four tubes on the left show complete precipitation, the fifth tube partial precipitation, and the sixth tube, which is the control, containing no spinal fluid, is unaffected.

and in the hands of the average technician are less likely to give false reactions.

Use and Interpretation of the Colloidal Tests. Precipitation of colloidal solutions by cerebrospinal fluid indicates that the fluid is abnormal, but the degree and pattern of the precipitation are not specific for any disease. The colloidal reactions should be interpreted as a part of the fluid picture, along with the pressure, cell count, globulin, serology and other indicated tests. In general colloidal tests are confirmatory in nature and helpful in differential diagnosis. The tests are most useful in the management of neurosyphilis. In paresis there is practically always a first zone precipitation, but first zone reactions are also present in other types of neurosyphilis and in many other diseases, of which the most important is multiple sclerosis. A first zone reaction in a patient with a negative blood and spinal fluid Wassermann is presumptive evidence for multiple sclerosis. A persistence of a paretic reaction after anti-syphilitic therapy suggests parenchymal neurosyphilis. A mid-zone reaction occurs in heterologous conditions such as meningitis, virus diseases, vascular lesions and tumors, and adds no information that could not be readily obtained by simple routine tests. In puru-

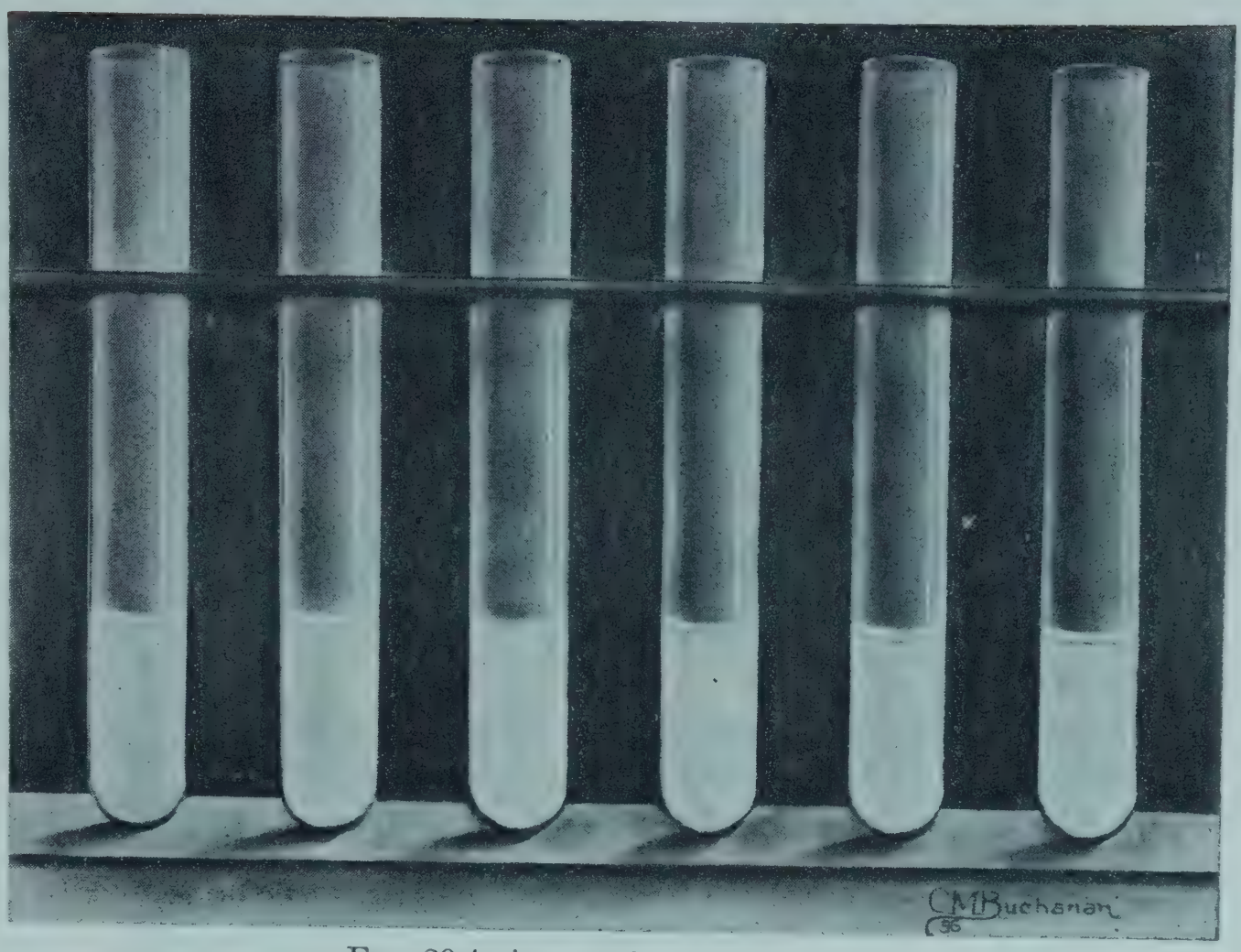


FIG. 20.4. A negative mastic test

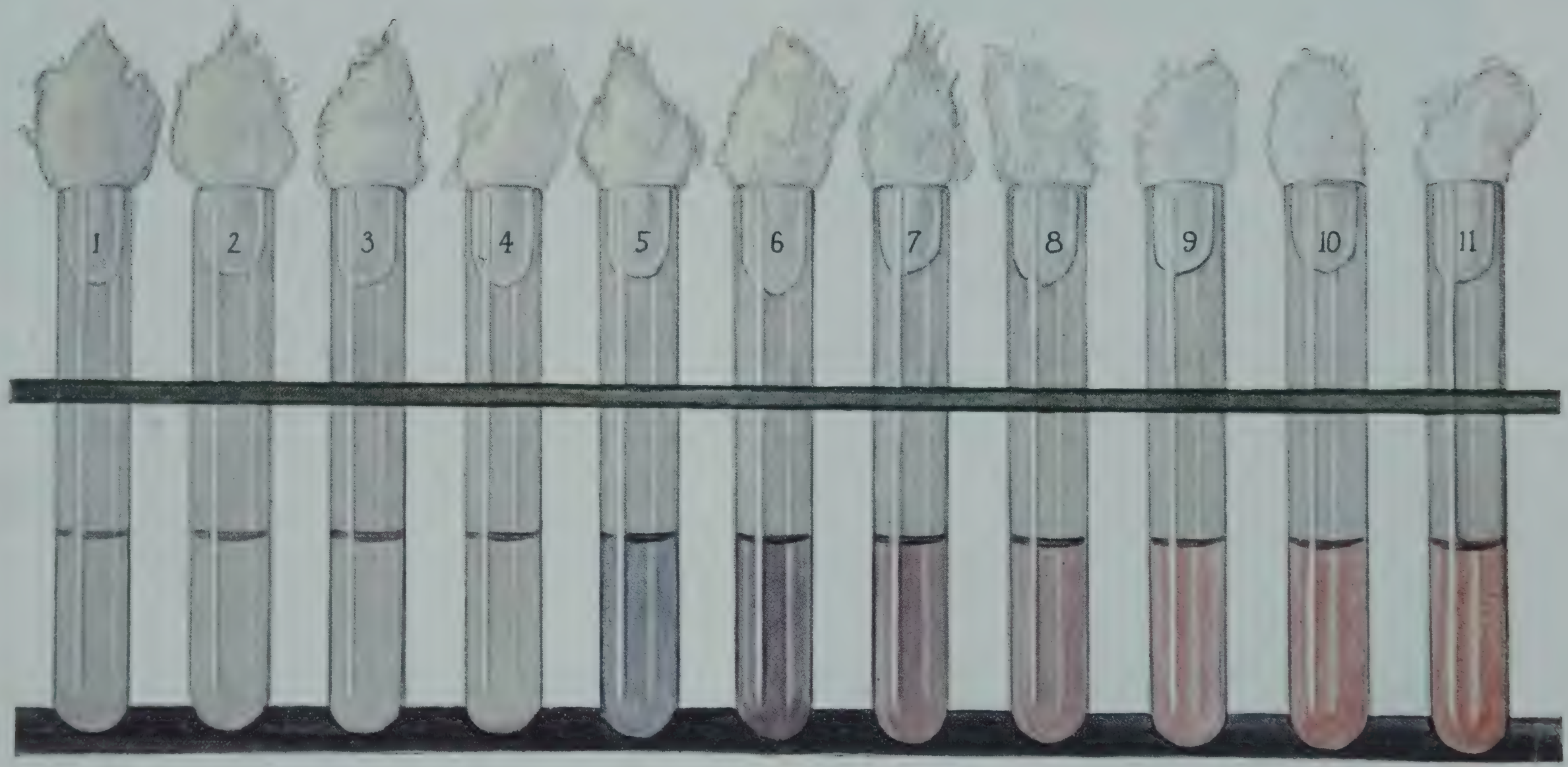
lent meningitis the reaction is in the third or end zone, but again the test is not necessary to make the diagnosis. In normal individuals slight reactions usually in the mid-zone may be obtained.

ALTERATIONS IN CEREBROSPINAL FLUID IN VARIOUS CONDITIONS

Children

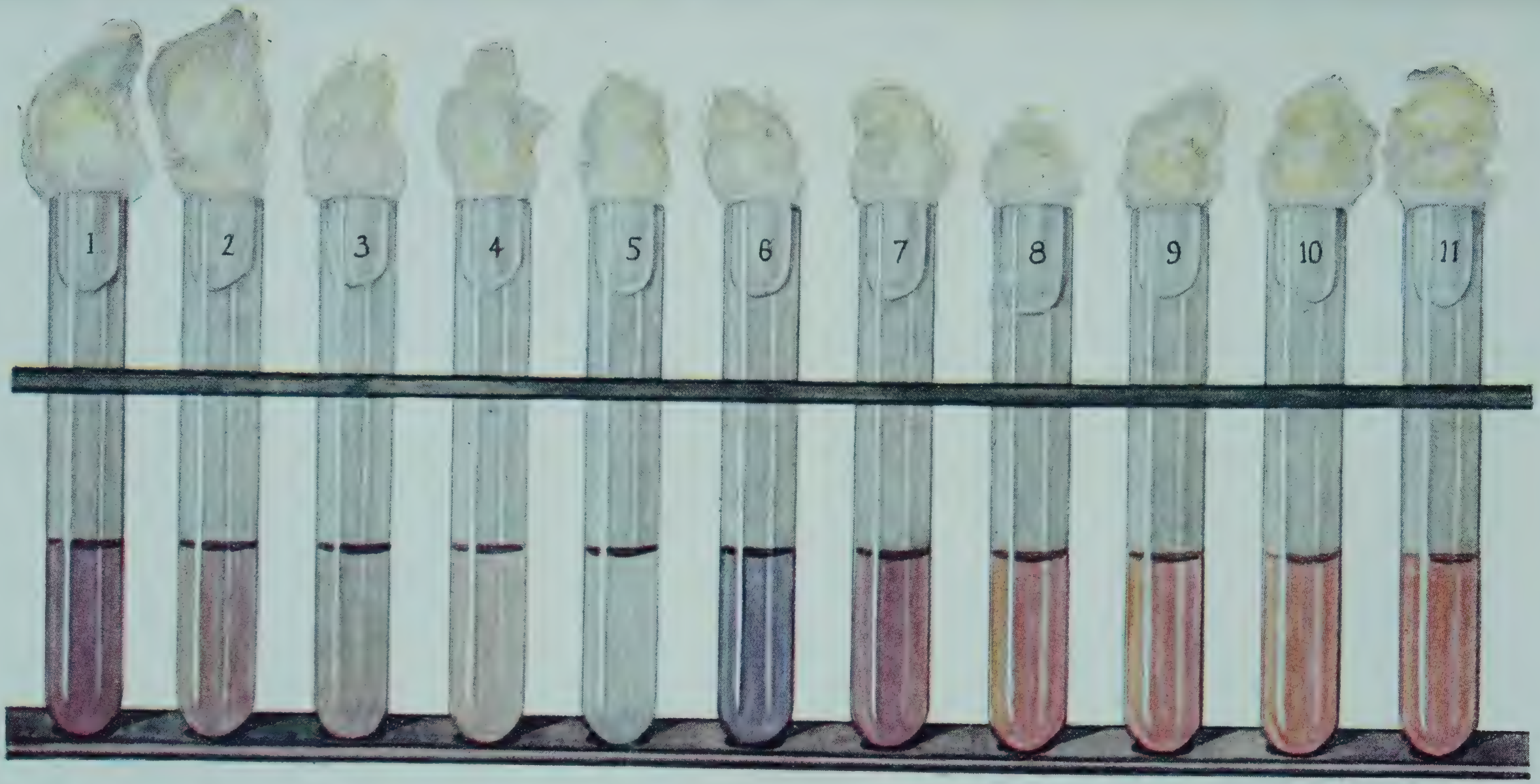
In infants lumbar puncture is usually performed with the patient sitting up, for in this position the child is easier to hold, the alignment of the needle is more accurate and the increase in spinal fluid pressure in the dural sac minimizes the danger of dural displacement and facilitates the collection of fluid. The ordinary 25 or 26 gauge hypodermic needle or the short Pitkin needle is recommended. Cisterna puncture is contraindicated as a routine procedure.

The normal fluid pressure in children in the horizontal position is about half that of adults, but manometric determinations are of little practical value for the patient usually resists the procedure or cries, thus giving false elevations. Bulging of the fontanelles and eye ground changes serve as indications of a marked increase in intracranial pressure. The range of cell count is higher in children than in adults, and counts of 10–20 are not to be interpreted as pathological unless supported



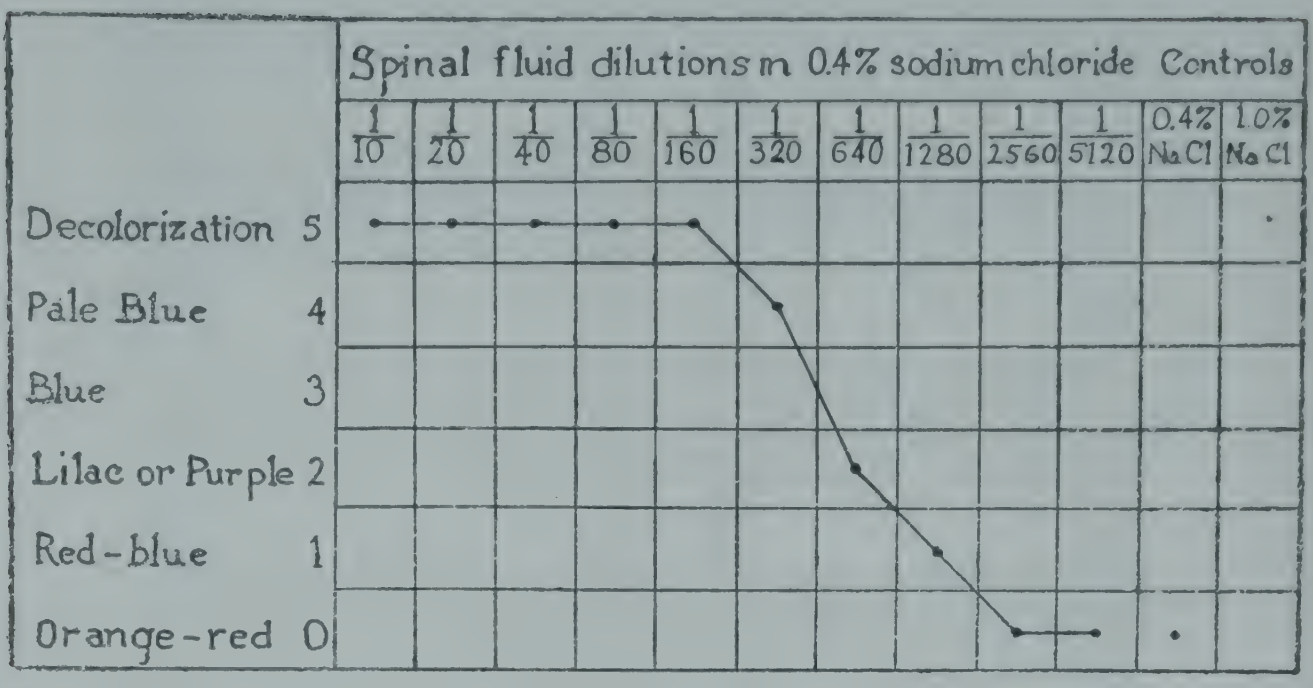
(Courtesy of Dr. Pasternack, U. S. Public Health Service

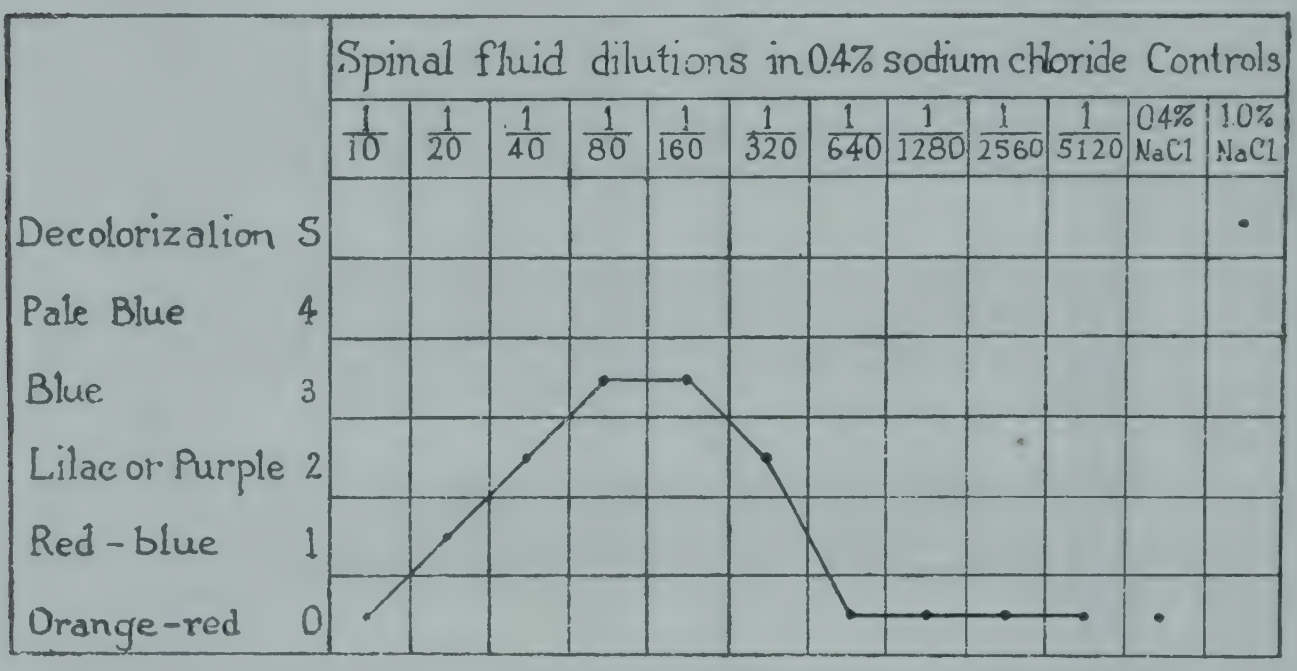
PLATE 20.I. Showing a typical positive reaction for paresis in a colloidal gold test



(*Courtesy of Dr. Pasternack, U. S. Public Health Service*)

PLATE 20.II. Showing a typical positive reaction for tabes dorsalis in a colloidal gold test. This is sometimes called a tabetic or luetic reaction.





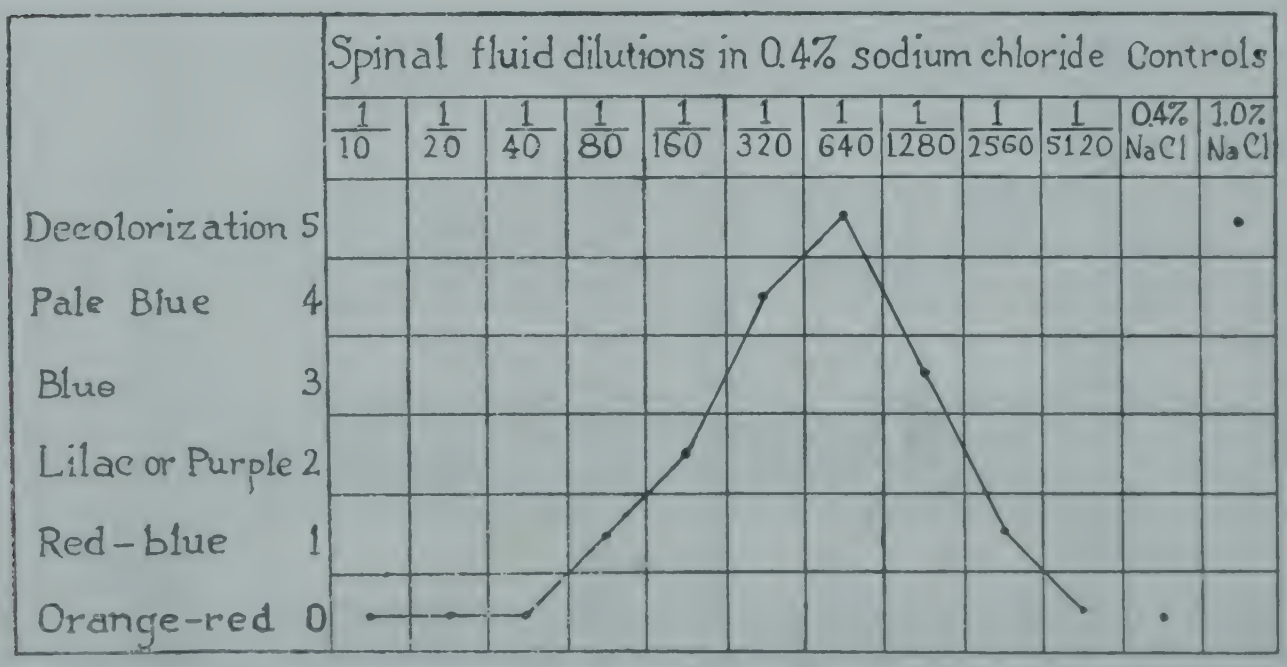


FIG. 20.5. Typical curves in various conditions obtained in the colloidal gold reaction. Top: A paretic curve (first zone curve). Center: A tabetic or luetic curve (mid zone curve). Bottom: A meningitic curve (third zone curve).

by other findings. Small quantities of traumatic blood are common. The diagnosis of subarachnoid hemorrhage in newborn infants from cerebrospinal fluid findings alone is hazardous, for there may be hemorrhage demonstrated at autopsy with a clear fluid containing only a few cells or many red cells may be present and no gross hemorrhage detectable at autopsy. Red cells associated with a xanthochromic supernatant fluid giving a positive benzidine reaction is diagnostic of ventriculo-subarachnoid hemorrhage. Xanthochromia is common in normal infants in the first few weeks of life, and is not necessarily related to hemorrhage or to clinical jaundice. The chemical and serological changes in children are essentially the same as in adults.

In infants it is easier to obtain spinal fluid than it is blood, hence for calcium, urea and other chemical determinations the spinal fluid may be substituted with advantage.

Blood in Spinal Fluid

Contamination of the cerebrospinal fluid with blood is usually due to a dull needle, too long a bevel, poorly fitting stylet, improper angle, or to advancing the needle too far. Blood due to needle trauma is likely to vary in amount in the collection tubes, being as a rule, most marked in the first tube and less in successive tubes. Traumatic blood coagulates. When the red cells settle, the supernatant fluid is colorless.

Since the cellular, antibody and chemical contents of the blood and spinal fluid differ widely, a mixture of blood with the fluid alters the findings. In general grossly bloody fluids are unsuited for tests other than culture. Fluids that are slightly cloudy or contain a few thousand red cells per cubic millimeter may be used for white cell count and corrections made depending on the amount of blood present. (See under Cell Counts.) The chemical tests on bloody fluid are run on the supernatant portion. Sugar and chloride determinations in cleared fluids are not significantly altered by moderate amounts of blood. For proteins, 4 mg. per 100 ml. is subtracted for each 5,000 erythrocytes per cu. mm. The presence of less than 5,000 erythrocytes per cu. mm. does not alter the colloidal reactions, but larger amounts will give false positives. A negative serological test is significant on bloody spinal fluid, but a positive test in the presence of a positive blood reaction does not mean neurosyphilis.

Conditions with Meningeal Signs

In the presence of headache, vomiting, stiff neck, hyperactive reflexes, mental, motor or sensory disturbances associated with fever and alter-

ations in the blood picture, the examination of the fluid by the more simple tests gives information of great differential value. The degree of fluid abnormality increases in direct proportion to the degree of inflammatory reaction.

The differential diagnosis between poliomyelitis, epidemic encephalitis and benign lymphocytic meningitis cannot be made from the cerebrospinal fluid findings. Occasionally in mumps, lympho-granuloma venereum, whooping cough, herpes, vaccinia, and in acute exanthemata there is meningeal irritation with increased pressure, lymphocytic pleocytosis and protein increase. In lead encephalopathy the fluid is as a rule clear, the pressure increased; there is moderate pleocytosis with a variable differential count, the protein is increased and the sugar and chlorides normal.

Aseptic meningeal reactions occur in brain abscess, in extra-dural infections secondary to mastoiditis and nasal sinus infections, lateral sinus thrombosis, septic cerebral embolus and necroses of brain tissue near the ventricle as in thrombosis, embolus, hemorrhage or tumor. The presence of a polymorphonuclear cellular reaction instead of a lymphocytic reaction is the principle differential point between this group of conditions and the virus diseases.

Neurosyphilis

The pathology of neurosyphilis varies in extent, in degree and in the type of inflammatory reaction. The anatomical differences between meningeal, vascular, or cerebrospinal types, gumma and degenerative lesions are not sharply drawn. Likewise the alterations in the cerebrospinal fluid in syphilis are characterized by variability, not only in different patients with different syphilitic syndromes, but in the same patient at different times. In using the cerebrospinal fluid findings as an aid in the detection of neurosyphilis, and in the appraisal of the underlying pathological process, the fluid picture is considered as a whole in correlation with the clinical findings and little reliance is placed on a single test. The tests most useful in syphilis are: *cell count, qualitative globulin, quantitative protein, serologic and colloidal reactions.* Measurement of pressure, differential counts, culture, sugar and chloride determinations are of little value in differentiating one type of syphilis from another, although they are essential in the differential diagnosis of neurosyphilis and non-syphilitic lesions. Pleocytosis, alteration in permeability of membranes with increase in proteins, positive serology and abnormal precipitation of colloids usually occur simultaneously and roughly paral-

TABLE 1

Diagnosis	Pressure Increase	White Cell Increase	Protein Increase	Chloride Decrease	Sugar Decrease	Other findings
Normal	0	0	0	0	0	0
Meningism	±	0	0	±	0	0
Encephalitis	±	+	±	0	0	Lymphocytes
Poliomyelitis	±	++	+	0	0	Chiefly lymphocytes
Benign lymphocytic meningitis	+	++	+	0	0	Lymphocytes
Localized or aseptic meningitis	+	++	+	±	±	Neutrophils predominant; no organisms
Syphilitic meningitis	+	++	++	±	±	Mostly lymphocytes; no bacteria; positive Wassermann
Tuberculous meningitis	++	++	++	+++	+++	Fibrin web; lymphocytes; few neutrophils; tubercle bacilli
Acute purulent meningitis	++++	++++	++++	++++	++++	Neutrophils; pyogenic bacteria

lel each other quantitatively, but this is by no means true in all cases, for numerous combinations of abnormality occur. Under treatment there is no uniformity in the sequence of disappearance of the abnormalities as reflected in the various tests, but the serologic abnormality is often the last to disappear.

Lumbar puncture should be done upon completing the treatment on early cases and if negative, repeated at the end of a year, followed by another spinal fluid examination at the end of the second year. Lumbar puncture should always be done before beginning treatment on latent cases and on all late cases. No patient should be discharged until the cerebrospinal fluid has been examined.

In primary and secondary syphilis the central nervous system is invaded by the treponema pallidum as are all other tissues and the spinal fluid is abnormal in 25 per cent or more of the cases, the findings varying from slight pleocytosis or positive globulin to formulas simulating acute meningitis or paresis. In latent syphilis the fluid may reveal abnormalities, and serve as a guide to treatment long before the lesions are extensive enough to become clinically manifest. In late neurosyphilis the abnormalities appear in proportion to the extent and degree of injury produced by the disease. The most marked changes occur in diffuse parenchymatous lesions (paresis) in which there is a definite pleocytosis (25–200 per cu. mm.), protein increase (75–200 mg.), positive Wassermann reaction using small quantities of fluid, and strong first

zone colloidal precipitations. The so-called paretic formula, is however, not diagnostic of paresis, but may be simulated by all other forms of neurosyphilis. Tabes, cerebrospinal and meningo-vascular syphilis present variable fluid patterns.

Vascular Accidents

Thrombosis, hemorrhage or embolus involving small blood vessels and in areas not in contact with the ventriculo-subarachnoid spaces produce little alteration in the cerebrospinal fluid picture. More extensive lesions cause meningeal inflammation, dilation of collateral vessels, edema, expanding mass and diapedesis of red cells or frank hemorrhage. In cerebral hemorrhage the fluid is grossly bloody in about three-fourths of the cases and the pressure is increased in about one half. In cerebral thrombosis gross blood is the exception rather than the rule, and the pressure is usually normal or only slightly increased. A manometric reading over 300 is common in cerebral hemorrhage and rare in cerebral thrombosis, and a pressure above 400 mm. practically excludes thrombosis. In spontaneous subarachnoid hemorrhage, the fluid is grossly bloody and under increased pressure. On removal of 10 to 15 ml. of fluid there is slight reduction in pressure, whereas in cerebral hemorrhage in which there is edema of the brain and an expanding mass which tends to collapse the ventricles and subarachnoid spaces, the removal of a small amount of fluid causes a marked drop in pressure. In subarachnoid hemorrhage the onset is usually with headache which increases in severity and is followed by meningeal reaction and unconsciousness hours later, whereas in cerebral hemorrhage or thrombosis the onset is sudden with convulsions, coma or localizing neurologic signs.

Cranio-cerebral Injuries

Trauma of the head produces a varied fluid pattern. Pressure increase, blood and pleocytosis are often found, but give unreliable diagnostic and prognostic information.

Brain Tumors

In brain tumor the pressure and proteins are usually increased, the cell count is variable but often normal, the sugar and chlorides normal, and the smears and cultures negative for bacteria.

Spinal Subarachnoid Block

Tumors of the cord or neighboring structures, fractures or dislocations of the vertebrae, epi-dural abscess, hemorrhage or aneurism cause a

closure of the spinal subarachnoid space and interefere with the circulation of blood and cerebrospinal fluid. The fluid in sacculated lesions is usually under increased pressure, but the volume of fluid is so small that the loss of a few drops or the escape of fluid into the manometer tube causes a marked reduction in pressure and hence a normal or low manometric reading. A comparison of the pressure in the lumbar and cisternal spaces taken simultaneously and the response to jugular compression are of aid in diagnosis. Roentgenograms taken after the injection of air or radio-opaque substances into the subarachnoid space aid in the location of the lesion. The fluid in spinal "block" is usually clear and xanthochromic. The cell count and differential are variable. In infections there is a neutrophilic pleocytosis. The protein is increased and clotting, soon after the fluid is removed, is often observed. The concentration of sugar tends to be high and the concentration of chlorides low.

Herniation of an Intervertebral Disc

Herniation of the nucleus pulposus causes pressure on the dura, partial occlusion of the subarachnoid space, interference with blood supply and irritation of the nerve roots (low back pain, tenderness over sciatic nerve and altered reflexes of the leg). The pain in about half of the cases is increased when the cerebrospinal pressure is increased by jugular compression or by coughing or sneezing. The protein is increased in about 75 per cent of the cases, and this is the most important and sometimes the only demonstrable abnormality. The protein determination should be made on the first specimen removed.

Epilepsy

In epilepsy of unknown origin the cerebrospinal fluid is normal or there is only a slight alteration in pressure, cell count and protein. In the presence of significant increases in pressure, cells or protein in a patient with convulsions, one should suspect brain tumor, abscess or vascular lesion rather than epilepsy.

Multiple Sclerosis

In multiple sclerosis the fluid findings are normal in most cases, but there may be a moderate lymphocytic pleocytosis, protein increase and a first zone colloidal reaction.

Miscellaneous

In migraine, polyneuritis, tetanus, botulism, chorea, paralysis agitans, subacute combined degeneration, muscle dystrophies, arteriosclerosis,

hypertension, psychoneuroses and numerous other conditions the fluid findings are normal, for these diseases produce little inflammatory reaction and are not associated with hydrostatic and osmotic abnormalities of a degree sufficient to interfere with the formation, circulation or absorption of fluid.

INTERPRETATION

The cell count and, if elevated, a differential count, are most important examinations and should be performed immediately. Elevation of the cell count is one of the earliest changes to occur in the spinal fluid indicating central nervous system disease. A lymphocytosis suggests syphilis, tuberculosis, a virus infection, tumor, or toxic irritant. A positive serologic reaction establishes the diagnosis of syphilitic infection. Demonstration of acid fast bacilli on smears, culture or guinea pig inoculation makes the diagnosis of tuberculosis possible. The Levinson or tryptophane tests are often helpful but not diagnostic of tuberculosis. Since the advent of curative therapy for tuberculous meningitis early intense efforts to demonstrate the organisms on smears should be made. Unfortunately, no further confirmatory studies to establish the diagnosis of a virus infection are possible with the cerebrospinal fluid, the virus rarely being present in the fluid and much less antibody is present than in the blood. Appropriate smears and cultures usually demonstrate the etiologic agent in purulent fluids.

The total protein is essential in most all cases. As has been emphasized, an elevated total protein may be the only significant alteration present. Accurate simple chemical methods are available and should be insisted upon. A bloody spinal fluid obviously greatly increases the amount of protein present and invalidates the test. A positive collidial test simply demonstrates the presence of an increased amount of gamma globulin. In the past far too much clinical diagnostic reliance unfortunately has been placed on these tests, accompanied by much worthless jargon. It is to be hoped that soon we will have a simple chemical technique for the albumin–globulin ratio in cerebrospinal fluid. Then quantitative reports of alterations in the protein fractions can be reported in milligrams and all the colloidal tests discarded.

The chemical determination of sugar, chlorides, etc., are frequently helpful adjuncts but are not in themselves diagnostic. Such information has to be considered carefully with the other cerebrospinal fluid findings and the clinical findings. The quantitative determination for alcohol is perhaps about the only exception.

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CHAPTER 21

RENAL FUNCTION TESTS AND URINE EXAMINATION

By Seward E. Miller

Renal Function Tests

GENERAL CONSIDERATIONS

Undoubtedly examination of the urine is the most frequently used of all clinical laboratory procedures. It is most useful in ascertaining evidence of disturbed function of the kidneys and pathologic lesions of the ureters, bladder, or urethra. In the male, lesions of the prostate and seminal vesicles also may be evidenced in the urine, particularly inflammatory lesions, and specimens of urine from females may be contaminated with material from the vagina.

A clear understanding of the mechanism of kidney function is essential for a complete appreciation of the significance of urinary findings and the renal function tests.

The kidneys, fortunately, have, like the liver, a tremendous reserve functional capacity. Normal life activities easily can be sustained by only one kidney and while many lesions, infections, tumors, or traumatic injuries may be unilateral, kidney disease generally is bilateral. The functions of the kidneys are: (1) to rid the body of waste products of metabolism, (2) to rid the body of foreign and nonendogenous substances, (3) to help maintain the water balance, (4) to maintain the acid-base equilibrium of the body.

The functional unit in the kidney is the nephron, composed of the glomerulus and its long tubule. The glomerulus, acting as a semipermeable membrane, allows the passage of water and solutes but retains protein and the cellular elements of the blood. The filtration pressure necessary to drive fluid across the membrane is that difference between the blood pressure of about 65 mm. of mercury and the osmotic pressure of protein which amounts to 35 to 45 mm. mercury. Thus, the rate of filtration is influenced by variations of blood pressure and concentration of plasma protein, while the character of the filtrate is influenced by the integrity of the membrane. In passage through the tubules the filtrate is concentrated by reabsorption of 98 to 99 per cent of the water. The dissolved solids are selectively *reabsorbed* in varying amounts according to each individual threshold value. Those sub-

stances such as glucose, amino acids, chloride, sodium, potassium, and calcium are reabsorbed to a great extent and are called threshold substances. Urea, uric acid, phosphates, and sulphates, reabsorbed less, therefore are concentrated in the urine to a greater degree and are called low threshold substances. Creatinine is not reabsorbed.

There are about one and a quarter million nephrons in each kidney and the bulk of the blood, normally about 90 per cent, coming to the kidney perfuses through these glomeruli. It has been estimated that over 150 ml. of filtrate which has the composition of protein-free plasma are formed each minute with the reabsorption of 98 to 99 per cent of the water.

The formation of urine involves three separate activities of the nephron: (1) filtration by the glomerulus, (2) selective reabsorption by the tubules, (3) secretion of foreign substances by the tubules.

The quantity of fluid ultimately reaching the bladder is 1200 to 1500 ml. during the 24-hour period and has the approximate composition given in Table 21.1. This composition is not constant, even in normal health, but varies with diet and fluid intake.

In diseased states the urine may contain abnormal amounts of these normally occurring constituents and in addition pathologic constituents such as proteins, sugars, acetone bodies, bile pigments, drugs and poisons, and certain formed elements as red cells, pus cells, casts, crystals, and bacteria.

Chemical examination of the urine for albumin, microscopic examination of the urinary sediment, and determination of the nitrogenous constituents of the blood furnish valuable information concerning the *late* pathologic changes in the kidneys. However, none of these tests determines early significant renal damage nor the functional reserve of the kidneys. To satisfy these needs, over fifty tests of renal function have been devised, of which certain ones are more satisfactory for general clinical use.

All three primary functions of the nephron can be measured by various tests, and in fact renal function tests are conveniently grouped into these three general classes—those testing (1) glomerular filtration, (2) tubular readsorption, and (3) tubular excretion.

The tests based on glomerular filtration and tubular reabsorption are the more physiologic, since these are the predominating functions of the kidney. The removal from the blood stream of foreign substances such as dyes by their excretion through the tubular epithelium is a reserve function.

TABLE 21.1

Average Normal Composition of Urine

(After Best and Taylor)

	Grams/Liter
Inorganic constituents:	
Chloride (as NaCl)	9.0
Phosphorus (as P_2O_5)	2.0
Total sulfur (as SO_3)	1.5
Sodium (as Na_2O)	4.0
Potassium (as K_2O)	2.0
Calcium (as CaO)	0.2
Magnesium (as MgO)	0.2
Iron	0.003
Organic constituents:	
Urea	25.0
Uric acid	0.6
Creatinine	1.5
Ammonia	0.6
Undetermined N	0.6
Sugar (insufficient for positive Benedict's test)	Trace
Ketone bodies	Trace
Carbonates, bicarbonates, and free carbonic acid	Trace
Pigments	
Mucin and mucin-like substances	
Diastase	

TESTS OF GLOMERULAR FILTRATION—THE CLEARANCE TESTS

Four clearance tests—urea, inulin, creatinine, and diodrast—have been used to examine for impairment of glomerular filtration. Urea clearance is more commonly employed in clinical examination and inulin and diodrast clearance for more meticulous experimental studies. Creatinine has been practically abandoned as a medium for clearance determination because of the lack of specificity of its color reaction.

Urea Clearance

The concept of renal clearance was defined by Van Slyke as the volume of blood which would be cleared of urea by one minute's excretion of urine. Van Slyke points out that the term "clearance" is a convenient way to picture the relationship between urea content of the blood and the rate of excretion, despite the fact that about 40 per cent of the

urea is reabsorption in the tubules and the blood is never completely cleared of urea.

It has been determined that the direct ratio between the blood urea content and the urine excretion rate remains constant regardless of the blood urea level when the urine volume is 2 ml. or more per minute, the so-called augmentation limit. With the normal adult kidney, this is equivalent to the excretion per minute of the amount of urea in 75 ml. of blood. This figure is called the maximum clearance (C_m). When the volume of urine falls below 2 ml. per minute the rate of urea excretion relative to the blood urea level decreases in proportion to the square root of the urine volume. This is equivalent normally to the excretion per minute of the amount of urea in 54 ml. of blood, which figure is termed the standard clearance (C_s). The figures for the maximum clearance (75 ml.) and for standard clearance (54 ml.) are considered 100 per cent kidney function and the findings in any given urea clearance test are expressed as the percentage of these figures. The normal range of function as given by Van Slyke) is 75 to 130 per cent.

Determination of Urea Clearance (Van Slyke). *Principle.* The urea clearance is calculated upon the basis of the blood urea concentration, urine urea concentration and rate of urine excretion by the use of formulas.

Preparation of Subject. No special preparation of the subject is necessary other than giving two glasses of water, one at the beginning of the test and one at the end of the first hour to promote free flow of urine. Except in advanced nephritis, the patient may either lie down or be up and about during the test.

Procedure:

1. Have the patient drink a glass of water and empty his bladder completely. This specimen is discarded. Accurately record the time of completion of urination.
2. At the end of approximately 1 hour have the patient void completely and again note accurately the time of voiding. Save this specimen.
3. Have the patient drink a second glass of water. Collect a blood specimen for urea determination.
4. At approximately the end of the second hour have patient void completely and accurately record the time of voiding. Save this specimen.
5. Measure accurately (within 1 per cent) the volume of urine in each specimen by using the smallest graduate cylinder that will completely contain the specimen. Calculate the per minute excretion of urine for each specimen by dividing the number of cubic centimeters by the number of minutes lapsed between voidings.

6. Determine the blood urea concentration and the urine urea concentration for each separate urine specimen.

Calculation:

If the per minute excretion of urine is 2 ml. or more, use the formula:

$$\frac{U}{B} \times V = C_m$$

If the per minute excretion of urine is under 2 ml., use the formula:

$$\frac{U}{B} \times \sqrt{V} = C_s$$

U = Urine urea mg. per cent
B = Blood urea mg. per cent
V = Volume of urine excreted per minute

$$\frac{C_m}{75} \times 100 \text{ or } \frac{C_s}{54} = \text{Per cent of normal clearance}$$

Inulin Clearance

Inulin clearance is a more specific test of glomerular filtration than the urea clearance since inulin is completely filtrable by the glomerulus and is not reabsorbed by the tubules. After the intravenous injection of approximately 25 gm. of inulin, the average adult clearance is 120 ml. of plasma per minute with the normal range from 100 to 150 ml. per minute. These figures are corrected, according to Smith, to a standard body-surface area of 1.73 square meters. This test, although admittedly superior for determination of glomerular filtration rate, has the disadvantage of requiring somewhat intricate chemical procedure for determination of the inulin content of blood and urine. Details of the method are given by Miller, Alving and Rubin. The procedure of the test is similar to that employed for urea clearance.

Diodrast Clearance

Diodrast is removed by the kidney through glomerular filtration with some tubular excretion but since it is completely removed and not utilized by the body it serves satisfactorily as a basis for clearance determinations. The material used is the same as that employed for intravenous pyelography. The normal diodrast clearance in the adult is 700 ml. of blood per minute. Normal limits are considered to be 520 to 1560 ml. per minute.

Interpretation of Clearance Tests

Diminished urea clearance is produced by glomerular destruction and also by certain extra-renal factors which decrease glomerular blood flow

or produce tubular back pressure. The relation between decrease in clearance and percentage of glomeruli destroyed is not direct, the percentage decrease of clearance being proportionally less than the percentage of non-functional nephrons up to 50 per cent destruction, and thereafter much greater.

In acute glomerulonephritis the urea clearance may range from normal to very low figures, depending upon the severity of the disease, and low clearance does not necessarily indicate poor prognosis. With recovery there is usually a beginning rise in the clearance. However, despite a return to normal of the clearance, the presence of continued urinary abnormalities, i.e. low specific gravity, casts, and red cells, as indicative of kidney damage and the renal disease may progress to a chronic form.

In chronic nephritis, steadily diminishing urea clearance indicates a poor prognosis whereas if the clearance stabilizes at a moderately low level (30 to 40 per cent), the outlook may be reasonably good for a number of years. Fixation of the specific gravity at about 1.010 usually occurs when the clearance has fallen to about 30 per cent. From this stage on in the progress of the disease no further changes in specific gravity can take place and therefore the urea clearance test is of most value in following the course and determining prognosis in severe chronic nephritis. When the clearance falls to 5 per cent uremia is either present or impending.

Since shock, severe dehydration, circulatory failure, and obstructions in the lower urinary tract diminish urea clearance, these conditions must be properly evaluated before low clearance values can be accepted as evidence of primary renal disease.

TESTS OF TUBULAR REABSORPTION—THE SPECIFIC GRAVITY TESTS

A number of tests have been devised to examine for impairment of the important function of tubular reabsorption. They are all based on the ability of the kidney (nephron) to concentrate urine. These tests measuring the tubular reabsorption (concentration) function of the nephron have been called, as a group, the specific gravity tests. They are extremely important, for the ability to concentrate urine is the first kidney activity to show significant impairment in nephritis.

These tests are conducted either under conditions of restricted fluid intake or by inhibiting diuresis by injection of posterior pituitary hormone. The latter methods as worked out by Sodeman and Engelhardt eliminate the delay in preparation of the patient. Of the numerous modifications of the former method (restricted fluid intake) available, those of Addis, Fishberg, and Lashmet and Newburgh being most com-

monly used. These require periods of fluid intake restriction preceding the period of urine collection. The specific gravity of specimens collected during this period is determined with a urinometer and correction made for albumin if present. For each 1 per cent of protein in the urine, 0.003 is subtracted from the observed specific gravity. Quantitative determination of albumin can be made by the method of Shevky and Stafford. For greatest accuracy in determining specific gravity, a Westphal balance and weighing bottles should be used.

Lashmet and Newburgh Concentration Test

This test imposes severe fluid intake restriction over a period of 38 hours and involves the use of a special dry diet for one day. Under these conditions normal individuals excrete at least one specimen of urine with specific gravity between 1.029 and 1.032, and a volume of urine averaging 1450 ml. for the first specimen and 36 ml. for each of the other two. Persons with renal disease show urine specific gravities ranging from 1.010 to 1.029, the degree of reduction of concentrating ability being influenced by the severity of the kidney damage.

Test Diet:

Food	Grams	Approximate Measure
Breakfast		
Cornflakes	15	½ cup
Bread (toast)	60	2 slices, ⅜ inch
Butter	20	2 squares
Cream 40%	50	¼ cup
Sugar	13	1 tablespoonful
Lunch		
Beef steak	100	4 x 4 x ½ inch
Potato (baked)	80	1 x 2½ inches diameter
Crackers	16	4
Butter	20	2 squares
Dates	70	10
Dinner		
Potato (baked)	80	1 x 2½ inches diameter
Lettuce	100	¼ head
Crackers	16	4
Butter	20	2 squares
Dates	30	4
Peaches (canned, no juice)	85	1 half

In addition to the above, 1 gm. sodium chloride (¼ level teaspoonful). No other salt is added in cooking or otherwise.

Procedure:

1st Day:

1. At 10 p.m. have patient stop all fluid and food for the duration of test except special test diet.

2nd Day:

1. Eat only special diet on this day.
2. At 8 a.m. empty bladder and discard urine.
3. Collect all urine voided after 8 a.m. on this day through 8 a.m. the next day in a single bottle and label specimen 1.

3rd Day:

1. No breakfast.
2. Collect urine at 10 a.m. and label specimen 2.
3. Collect urine at 12 noon and label specimen 3.
4. Determine specific gravity, volume, and albumin content of each specimen.

Comments:

1. Subjects may be allowed usual activity or bed rest.
2. Before specific gravity determinations are made, all urine specimens must be allowed to assume a uniform temperature at which all solids are in solution, preferably 25°C.
3. Urinometers must be calibrated at the proper temperature using the specific gravity of distilled water as 1.000.

Fishberg Concentration Test

This test imposes less strenuous curtailment of fluid intake and may be completed in a shorter period of time than that of Lashmet and Newburgh. With this procedure normal individuals excrete urine with specific gravity of 1.023 or higher.

Procedure:

1. The patient is allowed no fluids from 8 p.m. until 10 a.m. the next morning.
2. The evening meal is eaten about 7 p.m. It is high in protein and must have a fluid content of less than 200 ml.
3. Discard the night urine.
4. Collect urine specimens on arising, 8 and 10 a.m. and determine the specific gravity of each.

Addis and Shevsky Concentration Test

This test is longer and more tedious for the patient than the Fishberg, involving a longer period of abstaining from water, which, however, increases the sensitivity in detecting very early or more minor degrees of renal impairment.

Procedure:

1. The patient is allowed no fluids after 9 a.m. until 8 a.m. the following day.
2. A dry but otherwise normal solid diet is given.

3. All urine passed during the first 12-hour period 8 a.m. through 8 p.m. is is discarded.
4. After discarding 8 p.m. specimen, all urine voided during the night and the urine voided at 8 a.m. the next morning are saved as one specimen.
5. The specific gravity of this specimen should be greater than 1.026.

Concentration Test with Posterior Pituitary Extract (Sodeman and Engelhardt)

The subcutaneous injection of 10 U.S.P. units of posterior pituitary extract (1 ml. of obstetric or 0.5 ml. of surgical) in a normal person will inhibit the diuresis produced by the ingestion of 1600 ml. of water in 15 minutes. This action has been utilized in place of the usual period of fluid restriction to determine the concentrating ability of the kidney. The test has the advantages of a short performance time and elimination of the necessity for preparation of the patient. Posterior pituitary extract will also inhibit the diuresis seen in congestive heart failure under active treatment as well as that of diabetes insipidus, allowing sufficient concentration to determine degree of tubular function in these conditions. Sodeman and Engelhardt report no ill effects following the use of posterior pituitary extract with arteriosclerotic heart disease in congestive failure and with angina, but have not employed it in patients with marked oliguria, pregnancy, angina pectoris, or myocardial infarction.

Under the conditions of the test, individuals with normal kidney function excrete urine with specific gravities of 1.023 or higher. Failure to concentrate to this degree indicates renal damage.

Procedure:

1. The unprepared patient empties the bladder and the specimen is saved.
2. Inject subcutaneously 10 units of posterior pituitary extract (1 ml. of obstetric or 0.5 ml. of surgical).
3. The subject is given nothing by mouth until the test is completed.
4. Collect a specimen of urine at the end of the first and second hours.
5. Determine the specific gravity of each of the three specimens observing the usual precautions regarding temperature and correction for albumin.

Pasqualini has devised a different procedure for the test in which specific gravities are not determined but the volume of urine excreted is measured. The normal individual excretes 16 to 76 ml. of urine during the last hour of the test with the upper limit of normal being 80 ml. The excretion of a greater volume indicates impaired kidney function.

Interpretation of Specific Gravity Tests

It is important that specimens of urine kept in a refrigerator be brought to room temperature before measuring the specific gravity or that the

standard correction be made by adding one point to the last digit for each three degrees of temperature above 25°C. and subtracting one point for each three degrees below 25°C.

The concentration tests are of particular value in assaying early cases of nephritis since the inability to concentrate is the earliest manifestation of nephritis. As the disease progresses, diuresis as a compensatory measure is observed but finally the kidney is unable to produce dilute urine and the specific gravity of the urine becomes fixed at about 1.010. Progression of the disease beyond this point of severity can produce no further change of specific gravity, thus this type of function test is of greatest value in following the course of mild to moderately severe nephritis but gives no indication of progression or regression in the most severe cases.

All the specific gravity tests require cooperation on the part of the patient in the meticulous following of instructions. The Newburgh test requires hospitalization and careful supervision. The normal kidneys under conditions of this test excrete at least one of the specimens of urine with a specific gravity 1.029 or higher. Both the Fishberg and the Sodeman-Engelhardt tests use a specific gravity of less than 1.023 as indicative of impairment of renal function. Increasing impairment of function results in a corresponding reduction in ability to concentrate until with marked impairment of function the specific gravity becomes fixed at about 1.010.

TESTS OF TUBULAR EXCRETION

The reserve function of secretion of foreign nonendogenous materials by the tubular epithelium is most conveniently tested for by the use of certain dyes and measuring their rate of excretion. This type test is particularly valuable in establishing unilateral kidney disease.

Phenolsulfonphthalein Excretion Test

The use of phenolsulfonphthalein (phenol red) to measure renal function was first introduced by Rowntree and Geraghty (1912). For many years it was considered a measure of glomerular filtration but Smith has shown that with the amount of dye employed, 94 per cent is *excreted* by tubular action and only 6 per cent by glomerular filtration. Thus the test must be considered to measure primarily tubular activity.

In its original two-hour form the test has a low level of sensitivity that makes it worthless except in advanced kidney disease.

Fifteen-Minute Phenolsulfonphthalein Excretion Test

The two-hour test was modified by Shaw, who gave the dye intravenously and collected urine specimens at 15-minute intervals for 1 hour and a final specimen at the end of 2 hours. The test by this method has been shown to be as sensitive as the urea clearance test when the amount of dye excreted in the first 15 minutes is taken as the criterion of renal function. When 1 ml. of phenolsulfonphthalein solution (6 mg.) is injected intravenously, normal kidneys will excrete 30 to 50 per cent of the dye during the first 15 minutes. Excretion of less than 23 per cent of the dye during this period, regardless of the amount excreted in 2 hours, indicates impaired kidney function. Since the 15-minute specimen is the one of primary importance, the other specimens are not collected except for the 1 hour specimen which usually is collected.

In urologic practice the phenolsulfonphthalein test is widely used to determine the function of each kidney separately. Here the appearance time as well as the rate of excretion of the dye is of importance. After intravenous injection the normal appearance time of the dye at the end of the catheters is 2 minutes or less and the rate of excretion from 1 to 1.5 ml. per cent per minute. Increase in appearance time and decrease in excretory rate indicate impaired function.

Reagents:

1. Phenolsulfonphthalein solution (1 ml. contains 6 mg.). The dye may be obtained in ampule form ready for injection.
2. Sodium hydroxide solution—10 per cent.

Procedure:

1. Have patient empty the bladder and drink 600 ml. of water.
2. Thirty minutes later, inject intravenously exactly 1 ml. of phenolsulfonphthalein solution (6 mg.).
3. Collect a specimen of urine at 15 and 60 minutes after injection.
4. Determine the phthalein content of each specimen separately as follows: Add a few cubic centimeters of 10 per cent sodium hydroxide solution to the urine specimen to develop the color and dilute the specimen to 1000 ml. in a graduate cylinder. Compare the diluted specimen with artificial standards or in a colorimeter against a freshly prepared standard solution.

Comments:

1. Ampules of phenolsulfonphthalein ready for use contain slightly more than 1 ml. Caution must be exercised to measure the exact amount in the syringe and not inject the entire contents of the ampule.

Methylene Blue Excretion Test

A solution of methylene blue may be injected intramuscularly or intravenously. The appearance time generally is noted for each kidney

after catheterization of both kidneys through a cystoscope. The dye appears in about 30 minutes following intramuscular injection and in about 5 minutes after the intravenous injection.

Indigo-Carmine Excretion Test

The primary value of this test, as with the methylene blue test, is its usefulness in measuring the individual kidney function during cystoscopic examination. Four ml. of a 4 per cent solution of the dye are injected intramuscularly or intravenously. The normal appearance time is 10 to 15 minutes after the intramuscular injection and 3 to 5 minutes after the intravenous injection.

Interpretation of the Dye Tests

Reduced dye excretion may result from certain extrarenal influences, chiefly dehydration, shock, and circulatory failure, without the presence of actual kidney damage. Phthalein excretion based on appearance time and per minute excretion of dye is the test of choice for estimation of function in each kidney separately. As indicated, tubular excretion is probably a reserve function of the kidneys, thus the dye excretion tests show no abnormality in *early* kidney disease. In moderate and more advanced stages of impaired kidney function the phenolsulfonphthalein test generally roughly parallels the urea clearance test.

NON-PROTEIN NITROGENOUS CONSTITUENTS OF BLOOD

The blood level of the non-protein nitrogen or any of its chief constituents, urea, uric acid or creatinine, serve as a general indication of kidney function, but do not detect early renal damage. Significant retention of these substances in the blood is not observed until renal function, as determined by urea clearance or specific gravity tests, has been greatly reduced.

Under normal conditions the blood urea nitrogen constitutes approximately 50 per cent of the non-protein nitrogen but with kidney damage urea is retained to a greater degree than other nitrogenous constituents and therefore the blood urea level rises faster proportionally than that of the non-protein nitrogen. The ratio between these two substances may be altered before significant increase in the blood levels of either occurs. Mosenthal and Bruger feel that such an alteration is an indication of early kidney damage which compares favorably with the results of the urea clearance test. The normal ratio is 44 or less and is computed

as follows:

$$\frac{100 \times \text{Urea N (mg. per cent)}}{\text{N.P.N. (mg. per cent)}} = \text{Urea ratio}$$

CHOICE OF RENAL FUNCTION TESTS

The seclection of a renal function test for use in any instance is influenced by three primary considerations—the type of information desired, the nature and severity of the kidney lesion under investigation, and the scope of laboratory facilities available. If a quick, rough estimate of severity of functional impairment is desired as in the case of known urinary tract obstruction or in the presence of clinical symptoms suggesting uremia, determination of the blood urea nitrogen is often sufficient. When great sensitivity is desired as in the detection of very early impairment of function or in the evaluation of recovery from acute nephritis, the specific gravity tests are of greatest assistance. In a definitely established nephritis of slight to moderate severity the specific gravity, urea clearance or fifteen minute phthalein excretion tests serve equally well, but in advanced chronic nephritis, progression or regression of renal impairment is best followed with the urea clearance tests. For, as has been previously pointed out, the specific gravity tests are useless as a source of further information after nephritis has progressed in severity to the point of specific gravity fixation at about 1.010. The phthalein excretion test appears to be less sensitive than the specific gravity test for detecting impairment of kidney function in the early stages of chronic nephritis. The results, however, generally parallel those with the urea clearance tests in the more advanced stages of kidney damage.

Under conditions where inadequate laboratory facilities prevent the use of tests requiring chemical determinations on blood and urine, entirely satisfactory results may be obtained by employing the specific gravity or the fifteen minute phthalein excretion tests. These utilize simple equipment and procedure within the scope of any laboratory or physician's office.

Relatively little additional pertinent information is gained from the more prolonged, more difficult, and more trying for the patient, concentration tests. The Mosenthal test is now of only historic interest. The Lashmet and Newburgh test also is used infrequently. The more simple tests, such as the Fishberg concentration test, are most frequently used—often in conjunction with the phenolsulfonphthalein test. In the usual forms of renal disease specific gravity fixation occurs when the urea clear-

ance is about 15 per cent of normal and the phenolsulfonphthalein secretory function about 20 per cent of normal.

Examination of Urine

GENERAL CONSIDERATIONS

The mechanism of urine formation has been discussed along with the more precise renal function tests. However, the usual physical, chemical, and microscopical examination of the urine frequently furnishes much valuable information concerning kidney disease, metabolic disturbances, and other pathologic lesions, not only of the kidneys but in the ureters, bladder, and urethra. In addition, some genital lesions are responsible for abnormal findings in the urine.

COLLECTION OF SPECIMEN

Type of Specimen

Two types of urine specimens are more frequently used—the occasional or single specimen and the 24-hour specimen. The *occasional specimen*, urine from a single voiding, may be collected at any convenient time but preferably the specimen is passed immediately after arising in the morning. Urine voided at this time is more concentrated and the formed elements are usually in a better state of preservation. This type of specimen is adequate for routine qualitative examination.

A *24-hour specimen* is necessary for all quantitative examinations, since neither the normal nor pathological constituents are excreted uniformly during the 24 hours. The 24-hour specimen consists of all urine voided during the designated period and is collected in the following manner:

Collection of 24-hour specimen:

1. Have patient empty the bladder at the beginning of the collection period which may be any convenient hour (8 a.m.). *Discard this specimen.*
2. Collect *all urine voided* during the next 24 hours including the last specimen at 8 a.m. the following day.
3. During the collection period the urine must be kept at refrigeration temperature or a preservative used.

A night and day specimen is sometimes necessary, particularly when knowledge regarding the volume of urine voided during the night is desired. The method of collection is as given for the 24-hour specimen except that the urine is pooled as two specimens, one representing that

voided from 8 a.m. through and including a specimen at 8 p.m., and the second, that voided from 8 p.m. through and including a specimen at 8 a.m.

Catheterized specimens are not necessary from males or in general from females for routine examination. If urinalysis is required during the period of menstruation or at any time if cultures are desired, catheterization is necessary.

Any clean but not necessarily sterile bottle of at least 4-ounce capacity is a suitable container for the ooccasional specimen. Twenty-four-hour specimens are collected preferably in a bottle of sufficient capacity to hold the entire specimen.

Preservation. Examination of occasional specimens should be made as soon as possible after voiding. Bacteria proliferate rapidly with resulting ammoniacal decomposition and degeneration of the formed elements such as red cells, pus cells, and casts. *Refrigeration* is the preferable form of preservation and must always be used when the urine is to be used for culture or animal inoculation. A number of chemical preservatives are available, no one of which is entirely satisfactory for all types of examinations. The kind of preservative used should be written on the specimen label. Of those now in use the tablet recommended by the life insurance companies enjoys the most extensive use. Those in common use are:

1. Toluene, sufficient to form a thin film over the surface of the urine, is the best preservative for chemical constituents.
2. Formalin, 2 drops per ounce of urine, is most satisfactory for preservation of the formed elements. This will cause a false positive test for sugar.
3. Thymol, 0.1 gm. to 100 ml. of urine, is a good general preservative but may give a false positive test for albumin.
4. Boric acid, 5 grains to 4 ounces of urine, will inhibit bacterial growth but not growth of yeasts.
5. The Association of Life Insurance Directors recommend the following formula which is prepared in tablet form and used 1 tablet to 1 ounce of urine:

Monopotassium phosphate	0.100 gm.
Sodium benzoate	0.050 gm.
Benzoic acid	0.065 gm.
Urotropin	0.050 gm.
Sodium bicarbonate	0.010 gm.
Red mercuric oxide	0.001 gm.

Chloroform is not a satisfactory preservative because it gives a false positive sugar reaction and makes the removal of sediment difficult.

PHYSICAL EXAMINATION

Volume

About 1 per cent of the glomerular filtrate is excreted as urine. This results in an average 24-hour urinary output of approximately 1500 ml. with a night volume of one-half to one-fourth the day volume. The volume of urine formed is directly the result of a balance between glomerular filtration and tubular reabsorption and therefore will be altered by pathologic changes in the nephron. These activities, however, are subject to numerous extrarenal influences, the degree of tissue hydration or dehydration, efficiency of circulation, the concentration of blood proteins and certain electrolytes, and the activity of the posterior pituitary.

Polyuria or increased formation of urine occurs after the ingestion of large amounts of fluids, in diabetes mellitus and diabetes insipidus, and in the early stages of chronic kidney disease. The polyuria is probably a compensatory mechanism attempting to keep the blood cleared of waste products.

Nocturia or increase of night urine volume to 500 ml. or more with a specific gravity of less than 1.018 is frequently the first evidence of disease. Mercurial and xanthine diuretics and ammonium chloride produce polyuria by inhibition of tubular absorption.

Oliguria or decreased formation of urine follows restriction of fluid intake, dehydration from vomiting, diarrhea, fevers, or any other cause. Oliguria also is associated with heart failure, acute glomerulonephritis and the end stages of chronic glomerulonephritis, nephrosclerosis, and the various forms of suppurative kidney disease. Tubular degeneration as seen after sulfonamide administration, following hemolytic transfusion reactions, and in shock which may result in severe oliguria, in which 30 ml. or less of urine is excreted in 24 hours, or even anuria.

Anuria or complete suppression of urine formation may result from severe dehydration, shock, toxic, or anaphalactic reactions.

Specific Gravity

The specific gravity of urine is a measure of the relative amount of solids in solution and therefore is indicative of the degree of tubular reabsorption or concentration by the kidney. Under conditions of normal metabolism and renal function the specific gravity varies inversely with the volume of urine excreted. This relationship does not hold in certain disease states, as in diabetes mellitus where large quantities of urine of

high specific gravity may be excreted and in terminal nephritis where reduced urinary output is ascisoated with relatively lowspecific gravities.

Under average normal conditions the specific gravity varies between 1.015 and 1.025 but under conditions of high fluid intake or marked fluid restriction the normal kidney is capable of excreting urine with specific gravity as low as 1.001 or as high as 1.030. With renal damage there is first inability of the kidney to excrete urine of high specific gravity (loss of concentrating power) followed by loss of ability to dilute, resulting in fixation of the specific gravity at or near 1.010. Thus it is possible with a single specimen of urine of high specific gravity to rule out the presence of significant kidney damage.

Determination of Specific Gravity:

A urinometer (Fig. 21.1) is required. Fill the tube nearly full of urine and put in the float, taking care that the float does not touch the sides of the tube. This instrument is calibrated for readings at 22.5°C. For greater accuracy add 0.001 to the reading for each 3°C. above this temperature and subtract 0.001 for each 3°C. below. If there is insufficient urine to float the urinometer, the specimen may be diluted with two or three volumes of distilled water and the specific gravity determined on the mixture. Multiplication of the last two figures of the observed specific gravity by the dilution factor gives the approximate specific gravity of the undiluted urine.

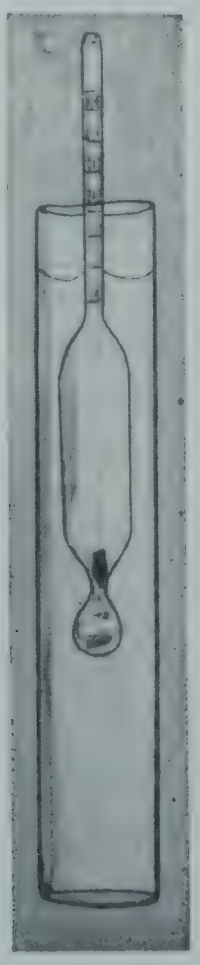


FIG. 21.1. A urinometer for testing specific gravity of urine. Note the curve of the meniscus.

Odor

Normal fresh urine has a faint aromatic odor but all urine upon standing undergoes decomposition and assumes an unpleasant ammoniacal odor. Freshly voided urine may have a putrid odor from extensive infection of the urinary tract. Certain drugs, as copaiba, saffron and turpentine, impart their specific odor, and some foods, particularly asparagus, impart characteristic odors.

Turbidity

Freshly voided acid urine is clear. However, it may be cloudy when alkaline due to the presence of phosphates. Pus and blood may cause turbidity, the former being white while the latter produces a red-smoky appearance. Upon standing urine may show clouding to the point of sediment formation from bacterial decomposition or precipitation of alkaline salts, mostly amorphous phosphates, or white to pinkish sediment of amorphous urates in acid urine. A faint cloud of mucus, leukocytes and epithelial cells may become visible with cooling. All these must be differentiated by microscopic examination.

Color

Under normal conditions the color of the urine varies directly with the concentration because *urochrome*, the chief normal pigment, is a product of endogenous metabolism, possibly dervied from muscle, and is excreted in constant quantities independent of the diet. Another yellow pigment which is normally present in the urine in traces is *urobilin*, derived from the urobilinogen formed in the intestinal tract and absorbed into the blood stream. With increased blood destruction, as in pernicious anemia or hemolytic icterus, increased quantities are formed, resorbed and escape the liver into the general circulation and are excreted in the urine. In liver damage increased amounts of urobilin may likewise appear in the urine. A positive test for urobilin, therefore, indicates increased blood destruction or pathologic changes in the liver (see Chapter 7, Liver Function Tests).

Other pigments (see Table 21.2) that may color the urine include *bilirubin* or its oxidation product *biliverdin*. Bile pigments are present in the urine only when some obstruction within the capillaries or duct system prevents their excretion by the liver. *Hemoglobin*, unaccompanied by red cells (*hemoglobinuria*), may appear in the urine in any state of excessive hemolysis. *Blood* in urine (*hematuria*), depending upon

the amount and state of preservation, may color the urine from smoky red to dark brown.

CHEMICAL EXAMINATION

Normal Composition

Although about 98 to 99 per cent of the water in the glomerular filtrate is reabsorbed in the tubules, it still constitutes about 95 per cent of the urine. The other 5 per cent (see Table 21.1) is 2 per cent urea, and the remaining 3 per cent is divided in small amounts between a number of inorganic and organic substances—principally sodium chloride, potassium, calcium, magnesium, ammonia, uric acid, phosphates, sulphates, and creatinine. Variations in diet, work, and exercise influence the relative amounts of these various constituents to a considerable degree, and with some variation in the total solids. Also, as previously indicated, the chemical composition of an individual's urine varies during different portions of the day in relation to meals, work, and sleep. Normally the total solids excreted in the urine vary from 60 to 75 gm. with no sugar and no protein. The excretion of solids also varies with age, gradually decreasing from the middle of the fifth decade (about 45) on.

A rough but valuable estimation of the total solids can be made by the following technique:

1. The last two figures of the 24-hour specimen specific gravity × 2.33.
2. This figure × the number of ml. in the 24-hour specimen.
3. This figure divided by 1000 = total solids in gm.

Reaction

The acidity of the urine is influenced by the type of diet and also by various drugs. Most *fruits* and *vegetables* provide more basic than acid radicals; they have an alkaline ash. The excess salts rejected by the tubules on such a diet tend to make the urine alkaline. Alkaline drugs such as *sodium bicarbonate* or *potassium acetate* are similarly excreted with the production of an alkaline urine. *Proteins*, however, give rise to excess of sulfuric and phosphoric acids which are eliminated by tubular rejection. Consequently a *high protein diet*, or the *fasting state* in which body proteins are metabolized, results in an acid urine.

Determination of the Reaction:

Litmus Paper. Dip a strip of blue and a strip of red litmus paper in the urine. If acid, the blue paper changes to red. If alkaline, the red paper

changes to blue. If no color change is seen, the specimen is neutral. Not infrequently a "neutral" urine will change red litmus blue and blue litmus red because of the presence of both alkaline and acid phosphates. Such a reaction is termed *amphoteric*.

Hydrogen Ion Concentration. The most important drugs used with urinary infections to make the urine acid are the ammonium salts of strong acids, such as hydrochloric, phosphoric and mandelic. Following ingestion and adsorption the ammonium radical is detached and converted to neutral urea by the liver, liberating the free acid, which, in turn, is promptly rejected by the tubules. The maximum urinary acidity than can be attained is about pH 5, because of the production of ammonia by the renal epithelium. This mechanism seems to operate as follows: As the tubular fluid gets more strongly acid the activity of the ammonia mechanism is increased so that in the vicinity of pH 5 production of ammonia is equivalent to rejection of acid.

In nephritis the excess acid is not so completely neutralized by am-

TABLE 21.2

Abnormal Color of Urine

Substance	Color	Pathologic States Occurring in
Bilirubin	Yellowish foam	Obstructive lesion of bile capillaries or duct system
Biliverdin	Greenish foam	Oxidation product from bilirubin
Increased urobilin	Yellow to amber	Increased blood destruction or pathologic changes in liver
Porphyrins	Reddish	Congenital porphyria, lead and barbital poisoning, pernicious anemia, and hemolytic jaundice
Hemoglobin	Clear red to reddish-brown	Paroxysmal hemoglobinuria, malaria, transfusion reaction
Blood	Smoky red to brown	Acute nephritis, infarcts in kidneys, bleeding lesions in urogenital tract
Melanin	Brown to black	Malignant melanoma, leukemia, ochronosis, and carcinoma of liver
Homogentisic acid	Black	A congenital anomaly, alkaptonuria
Methylene blue	Blue	Administered for renal function test
Pyridium	Orange to red	Administered as urinary sedative
Acriflavine	Greenish fluorescence	Administered as urinary antiseptic
Phenol	Dark, smoky	Poisoning
Phenolsulfonphthalein	Red in alkaline urine	Administration as laxative or for renal function test

monia, resulting in urine a little more acid than normal. Acid urines in diabetes result from excretion of hydroxybutyric and diacetic acids.

Determination of Hydrogen Ion Concentration:

Nitrazine Paper. For the determination of the pH of the urine sufficiently accurate for clinical purposes over the range of 4.5 to 7.5, "Nitrazine" paper is most useful. A strip of this paper is moistened with the urine and then compared with the color chart which accompanies the package.

Estimation of Total Acidity. Estimation of the total acidity is of occasional value. The Folin-Wu method is reported as the calculated number of ml. of N/10 sodium hydroxide required to neutralize 100 ml. of urine, normal 25 to 40 ml., or for a 24-hour specimen, normal 554 to to 664 ml.

Chloride

Urinary chloride levels are of significance in several conditions, particularly in the clinical management of hypertensive and cardiac patients on low salt diets and certain pre- and post-operative cases in helping determine the type of fluid needed. Heretofore the Volard-Harvey chemical technique was somewhat of a deterrant to the routine use of urinary chloride findings. A simplified tablet test is now available which is sufficiently accurate for clinical guidance and has the added advantage of being carried out at the bedside in the office or home.

The tablet, containing silver nitrate and buffers when added to urine dissolves, and the silver ion combines with the chloride ions forming insoluable silver chloride. The 130-mg. tablet reacts with 0.09 mg. of chloride, with the formation of a brown to reddish precipitate indicating the end point. The amount of chloride present then is calculated. 0.09 mg. of chloride is the equivalent of 0.15 mg. of sodium chloride with about 9 gm. sodium chloride per liter an average normal.

Urea

Urea is the chief form in which the nitrogenous wastes of the body are excreted. It makes up 80 to 90 per cent of the total nitrogen of the urine, the average daily excretion varying from 25 to 35 gm. Increased amounts are associated with excessive protein catabolism. Little significance may be attributed to these figures unless the protein intake is accurately determined. Decreased excretion is associated chiefly with impaired kidney function but even here the findings must be related to the blood urea level. The amount of urea nitrogen present in urine may

be determined using the same technique as for blood urea nitrogen (see Chapter 7, Blood Chemistry). The only modification necessary is to dilute the urine 1:100. The report is calculated in mg. urea N per 100 ml. of urine. The 24-hour urinary output should be measured and the daily urea N excretion calculated.

Ammonia

Ammonia in combination with acid radicals is present in normal urine to the extent of approximately 0.6 gm. per liter and represents 2 to 3 per cent of the total nitrogen. Ammonia appears to be formed in the kidney largely from glutamine and to a much smaller degree by deamination of amino acids. Its formation by the kidney is one of the means for conservation of fixed base which allows acid bodies to be excreted as ammonium salts with the return of sodium to the blood. Urinary ammonia is increased in any condition where large quantities of acid bodies are cleared through the kidneys as in diabetic acidosis.

Uric Acid

Uric acid is present in the urine in the form of sodium, potassium, and ammonium urates as well as in free form, and in concentrated urine these urates are precipitated as amorphous urates. The amount of urates normally excreted is approximately 0.6 to 1 gm. per 24 hours. Increases are associated with the same pathologic conditions that influence the blood levels (see Chapter 7, Blood Chemistry). In nephritis there is a decrease in the urinary uric acid concomitant with its retention in the blood. Clinically, the occasion requiring a quantitative determination seldom arises, since the blood uric acid levels are preferred. If, however, a urinary uric acid determination is desired, the method of Benedict and Franke is recommended.

Creatinine

Creatinine, being of endogenous origin, is excreted in the urine in a constant amount, averaging approximately 1.25 gm. per 24 hours. The excretion is not influenced by diet but is related to muscular development and activity. Thus the amount eliminated in men is generally greater than in women. The creatinine coefficient, an expression of this relationship, is from 20 to 26 for normal men and from 14 to 22 for women. It is determined as follows:

$$\frac{\text{Mg. of creatinine excreted per 4 hours}}{\text{Body weight in kg.}} = \text{Creatinine coefficient}$$

Creatine

Creatine, the precursor of creatinine, under normal conditions is present constantly in the urine of children (10 to 50 mg.), occasionally in the urine of women, but rarely in the urine of men. Infantile creatinuria, about the age of puberty, ceases abruptly, or in the female passes over into the adult form. Creatinuria of women is intermittent, appearing in relation to the menstrual cycle, and is not exhibited by all women. Unexplained increases of urinary creatine commonly occur during pregnancy. The quantity eliminated rises slowly during the last trimester, reaches a maximum shortly after delivery, and subsides quickly in 12 to 14 days. Creatinuria is of no known clinical significance.

Protein

Protein in the urine is termed *albuminuria* and is mostly albumin derived largely from blood plasma. It is probably due to increased permeability of the glomerular filter. It is present normally in traces too small to be detected by the usual tests. Although the protein is chiefly albumin it is accompanied by small quantities of globulin. The greater quantity of albumin is apparently due to its relatively small molecular weight (70,000) as compared to the larger molecule of globulin (165,000). Proteins with smaller molecular weights such as hemoglobin (68,000) and Bence-Jones protein (35,000) are excreted with relative ease. Thus the normal glomerular filter is considered to be impermeable to proteins with a molecular weight of 70,000 or greater. However, the work of Addis suggests that an appreciable amount of protein may pass the glomerular filter and be reabsorbed by the tubules.

The permeability of the glomerular filter seems to be increased by any factor tending to cause local or generalized asphyxia. Therefore, albuminuria may be caused by a variety of circulatory disturbances, such *violent exercise*, *torsion on the renal blood vessels*, or by *heart failure*. Intermittent or postural or *orthostatic* albuminuria is reported to occur in 5 to 16 per cent of young individuals, being seen more frequently in thin, lordotic females. In this condition albumin appears in the urine after standing or sitting and disappears on reclining. Transitory venous congestion is thought to be the cause and orthostatic albuminuria should not be interpreted as indicative of present or impending kidney disease. In *febrile conditions* a slight albuminuria very frequently occurs. *Various drugs* such as turpentine or mercury cause renal irritation and albuminuria.

Albumin is found in the urine both in kidney disease and tumors in the urinary tract associated with hematuria. Albuminuria is variable with renal tuberculosis, amyloidosis, and suppurative kidney diseases.

Detection of albuminuria is of importance in the diagnosis of nephritis. In nephrosis and amyloidosis the albuminuria may be marked. A very marked albuminuria is also observed in hemorrhagic glomerulonephritis during the acute and active stages, while in the terminal stage with secondary contracted kidneys it diminishes considerably and during latent stages it may clear up entirely. In nephrosclerosis the albuminuria is variable. Only a trace is found with the benign hypertensive type, while with malignant hypertension the albuminuria may be heavy. Heart failure may occur in arteriosclerotic nephritis and distort the urine findings. Long continued or unusually marked albuminuria results in depletion of the plasma and tissue proteins with the production of marked edema. Massive antibiotic therapy has produced false positive tests for albumin in the urine.

Qualitative Determination of Albumin. Detection of albumin in urine is based upou the precipitation of protein from solutions by heat, strong acids or heavy metals. Cloudy urine should be cleared by either centrifugalization or filtration before being tested. The supernatant urine obtained by centrifuging for microscopic examination may be used after decanting from the sediment.

1. *Heat and Acetic Acid Test.* Fill a test tube three-fourths full of *cleared* urine. Hold the tube at the bottom with a test tube holder and boil the upper portion for about a minute in the flame of a small Bunsen burner or alcohol lamp. Agitate the tube while heating to prevent boiling over. If albumin is present, a cloud (Fig. 21.2) varying in intensity with the amount present will appear. Phosphates also produce a cloud upon boiling which clears up upon the addition of (1 to 5) drops of glacial acetic acid. If the cloud is due to albumin, it will be intensified.* A faint cloud due to only a trace of albumin can be seen best by holding the tube so that it has a dark background a few feet away and has light (from a window or otherwise) falling upon it from the side (Fig. 21.2).
2. *Heller's Nitric Acid Test.* Place 3 ml. of concentrated nitric acid in a test tube. With a pipette allow urine to run slowly down the side of the inclined tube so as to form two layers of fluid. A white precipitate at the junction of the two layers denotes the presence of albumin. It is best developed in about one minute. This test is advantageous when only small quantities of urine are available.
3. *Albumin Test, Denco.* This new and rather novel technique requires no heat and no solutions. It employs a uniquely designed paper filter-funnel.

* Maximum precipitation of albumin is obtained at about pH = 4.5.



FIG. 21.2. Showing the cloud of a positive albumin test held before a dark background.

The paper filter cone impregnated with citric acid is inserted in the paper funnel which is coated with sulfosalicylic acid. They constitute a single unit. The procedure is simple, reliable, and most rapid.

a. Press and completely open paper filter funnel.
b. Insert in empty test tube.
c. Pour urine sample into filter funnel, using 2 or up to 5 ml. (the capacity).
d. The filtrate is clear unless albumin is present, when the filtrate is cloudy. The turbidity is proportionate to the amounts of albumin precipitated by the sulfosalicylic acid.
e. The results are recorded as negative, trace, small, moderate, or large amount.

4. *Robert's Test*

Reagents:

Nitric acid, conc	1 part
Magnesium sulfate, saturated solution	5 parts

Procedure:

Place 5 ml. of Robert's Reagent in a test tube. Incline the tube and from a pipette, allow urine to run slowly down the side of the tube so as to form two layers of fluid. A white precipitate at the junction of the two layers indicates albumin. This test is considered more delicate and less liable to false positive reactions than Heller's test.

Qualitative tests are reported according to the following criteria:

Negative	No cloudiness
Slight trace	Cloudiness or ring can just be seen against a black background.
1 plus	Cloud is distinct but not granular or flocculent.
2 plus	Cloud is distinct and granular with flocculation. About 0.1 per cent.
3 plus	Cloud is dense with marked flocculation. About 0.2 to 0.3 per cent.
4 plus	Heavy precipitate to solid coagulation. About 0.5 or higher per cent.

Quantitative Estimation of Albumin. There is no practical quantitative test for albumin that is absolutely accurate but Purdy's or a modified Tauchiya's are sufficiently accurate for practical purposes. A portion of urine from a well mixed 24-hour specimen should be used.

Bence-Jones Protein

This type of protein, the properties of which were originally described by Bence-Jones in 1848, appears in the urine frequently in multiple myeloma. Its occurrence has also been reported in a number of other conditions, notably hyperparathyroidism, bone tumors, leukemia and empyema, but critical examination of methods of detection suggest that in these diseases it may have been confused with other closely related proteins. Jacobson and Miller describe a pseudo Bence-Jones protein which differs from Bence-Jones protein in being insoluble at the boiling point with both a pH 5 and nitric acid. They also present a method of detection which specifically differentiates Bence-Jones protein from pseudo Bence-Jones protein, nucleoprotein, heat-coagulable protein, and proteose.

The identification of Bence-Jones protein is based on the properties of coagulation at pH 5 at 45° to 70°C., redissolving at 100°C. at pH 5

or with nitric acid, and reappearing when the temperature is again brought to 70°C. or below.

Sugar

Sugar in the urine or *melituria* is most commonly glucose (dextrose) but fructose, galactose, lactose, sucrose, pentoses, and on rare occasions other polysaccharides, may be present.

Glucose. The presence of glucose (dextrose) in the urine, or *glycosuria*, is dependent upon three factors: the concentration of the sugar in the blood, the rate of glomerular filtration, and the degree of tubular reabsorption. All three of these being normal, the amount of sugar present is too small for detection by the methods usually employed. When the blood sugar rises above 160 to 180 mg. per cent and renal function is normal, dextrose appears in the urine. This level is known as the *renal threshold* for sugar and varies slightly in different normal individuals. Unless there is some disturbance in the storage or utilization of glucose this level is usually not exceeded even after eating a large quantity of carbohydrate, which is converted to glucose in the alimentary tract. On the other hand, the blood sugar may be within physiologic limits but with tubular reabsorption inhibited glycosuria will be present.

Diabetes mellitus is the most important disease that causes glycosuria. This results from a faulty utilization and storage of sugar, either from a defect in production of the pancreatic hormone (insulin) or the inhibition of insulin by other hormones. Glycosuria is not constant, however, for the renal threshold at which glucose spills over into the urine may be considerably elevated in chronic diabetes, so that glycosuria is not regularly observed. Other diseases involving the pancreas, such as *hemochromatosis*, may cause glycosuria.

In *renal glycosuria*, sugar appears in the urine with blood sugar levels at or only slightly above the normal fasting range and may be observed after each meal. This is a benign condition with a hereditary tendence in which tubular reabsorption is inhibited. The condition does not progress to diabetes mellitus, which disease, however, must be ruled out by other studies (see Glucose Tolerance Tests, Chapter 7, Blood Chemistry).

Emotional excitement, *chilling*, and various conditions associated with *asphyxia* or *acidosis*, stimulating the adrenal sympathetic mechanism, may cause glycosuria. In *hyperthyroidism* this mechanism is so sensitive that sugar frequently is found in the urine.

Diseases of the pituitary, such as *acromegaly* and basophilic adenoma,

may be associated with glycosuria, perhaps due to excessive inhibition of the pancreatic hormone. A similar inhibition is apparently caused by various *infections.*

Injury to the floor of the fourth ventricle causes glycosuria. Glycosuria may also occur with increased *intra-cranial* pressure from cerebral hemorrhage or brain tumor.

Small quantities of glucose escape into the urine in various types of nephritis presumably because of impaired tubular reabsorption.

Qualitative Determination of Sugar. There are several tests that are satisfactory. The Fehling and Benedict tests are in widest use. The Fehling is usually required by insurance companies, although the Benedict is probably more accurate.

1. Fehling's Test. Two separate stock solutions are necessary—Fehling's alkaline solution (Solution A) and Fehling's copper sulphate solution (Solution B). These two separate solutions keep well, but when mixed together the resulting "test" solution does not keep more than a few days. Therefore, it is desirable to prepare the test solution daily or at the time a test is made by mixing together equal parts of the alkaline solution and the copper solution. The separate solutions should be kept in glass stoppered drop-bottles for ease and convenience of pouring the solutions from them.

Procedure. Place equal parts of solutions A and B into a test tube. This is Fehling's test solution. Heat the solution to boiling and then add about one-half as much urine and boil again. If sugar is present, the solution assumes an opaque yellow color and shortly after a dense yellowish-red sediment falls to the bottom. Shake the tube while heating to prevent "popping" of the mixture.

2. Benedict's Qualitative Test. This test is considerably more sensitive than Fehling's, and chloroform, creatinine, uric acid, and various drugs do not interfere so much as with Fehling's test. It is the more popular and widely used, although some workers regard its great sensitivity as an objection, since rarely traces of sugar occur in the urine of apparently normal individuals. However, with proper interpretation of results this sensitivity is more of an asset than an objection.

Procedure. To 5 ml. of Benedict's qualitative reagent in a test tube, add 8 drops of urine. Boil for one or two minutes. To prevent bumping and popping out, shake the tube well as it is being boiled or put in two or three glass beads. A better method is to place the tube in a boiling water bath. Place in a rack and allow to cool spontaneously.

If sugar is present a turbid green, yellow, or red color develops, depending

upon the amount of sugar present. A clear solution or a white turbidity due to precipitated urates is a negative test. The following scheme is recommended for use in reporting:

Negative—clear blue color
Trace—pale green color
1 plus—greenish precipitate
2 plus—green-yellow precipitate
3 plus—yellow-orange precipitate
4 plus—orange-red precipitate with no blue remaining in supernatant fluid after precipitate has settled.

Quantitative Estimation of Glucose. There are several different quantitative tests for sugar but Benedict's test seems most satisfactory. Benedict's quantitative solution is so made that 0.002 gm. (2 mg.) of glucose exactly removes the color from 1 ml. of this solution. Thus, after titration, the total amount of sugar present can easily be calculated. The quantitative test is most significant when performed on a well-mixed twenty-four specimen.

Fermentation Test. This test is useful in distinguishing non-glucose reducing substances and lactose from glucose. It depends upon the fact that glucose is fermented by yeast with the evolution of CO_2.

Fructose. Fructosuria or levulosuria appears to be due to a metabolic defect in which there is failure on the part of the liver to convert fructose to glucose as well as an inability to break down fructose to lactic acid. When persons having this defect ingest fruit sugar, cane sugar or honey a variable portion of the fructose content is excreted intact in the urine. Fructose may accompany glucose in diabetic urine.

Fructose is a ketose-hexose, levarotatory and, because of its ketone configuration, is a more active reducing agent than glucose.

Qualitative Determination of Fructose.

1. Rate of Reduction of Benedict's Qualitative Reagent:

1. Dilute the urine so that the concentration of sugar is 0.2 to 0.3 per cent and mix 1 ml. with 5 ml. of Benedict's qualitative reagent in a test tube.
2. Place in a water bath at 55°C. for 10 minutes. A yellow precipitate forming in 5 to 10 minutes indicates a ketose since an aldose will produce no reduction in that length of time.

Notes:

1. Indican in the presence of nitrites may produce a blue color sufficient to obscure the yellow of a positive test.
2. Santonin and rhubarb produce false positive tests.

Quantitative Determination of Fructose.

Benedict's Quantitative Test:

The procedure is the same as for glucose. In calculation, 25 ml. of Benedict's reagent is equivalent to 53 mg. of fructose.

Galactose. Galactosuria has been reported occurring in infants who appeared to have an intolerance for milk in which the ability of the liver to convert galactose to glycogen was impaired. In all probability these cases are primarily ones of hepatic dysfunction and the galactosuria is a consequence of a diet high in lactose.

Lactose. Lactosuria occurs occasionally in women late in pregnancy, during the nursing period, immediately after weaning and after a miscarriage, but is rarely seen under other circumstances.

Lactose is a disaccharide which reduces Benedict's reagent, is not fermented by yeast. The quantitative determination is performed as with glucose with 25 ml. of Benedict's quantitative reagent equivalent to 67.6 mg. of lactose.

Sucrose. Sucrose or saccharose is normally hydrolyzed in the intestinal tract to glucose and fructose and does not appear in the urine as such. Rare cases are reported in both adults and children of sucrosuria but it is probably the least common form of melituria. Individuals feigning illness occasionally put sucrose in their urine, but, being a non-reducing sugar, it is not detected by Benedict's reagent.

Pentoses. Pentosuria may be of the alimentary type, intermittent, associated with the ingestion of large quantities of certain fruits which are high in pentoses such as cherries and plums; or it may be primary, persistent, due to an inborn error of metabolism. The latter type has a strong hereditary tendency and has occurred more frequently in Jewish people. The condition is not related to diabetes mellitus and appears uninfluenced by diet. The sugar present is xyloketose which, in addition to giving a positive Bial's test for pentoses, reduces Benedict's reagent rapidly as with fructose.

In alimentary pentosuria, the sugar present is usually l-arabinose, an aldose-pentose.

Other Reducing Substances. A number of reducing substances other than sugars may be present in urine. Dextrins from ingested pastry, homogentisic acid, and glucuronates reduce Benedict's reagent, although the reduction by homogentisic acid is atypical, ultimately forming a black color. A non-fermentable reducing substance occurs in the urine of persons with myasthenia gravis. The urine of patients on strepto-

mycin and other antibiotic therapy may also give positive reduction tests.

Acetone Bodies. The acetone bodies are: acetoacetic acid (diacetic acid), acetone, and β-hydroxybutyric acid. A state in which these substances are present in increased amounts in the blood and urine is called *ketosis* and is one type of acidosis. The mechanism of ketosis described by Chaikoff and Soskin, and Lundsgaard, differs from that previously taught, which held that the acetone bodies represented an unburned residue of the fatty acid chain, complete combustion of which would have required a more vigorous "fire" of carbohydrate. It now has been determined that aceto-acetic acid and β-hydroxybutyric acid, from which acetone is derived, are normal intermediate products of fat metabolism formed in the liver and carried to the tissues for further oxidation. When the supply of glycogen in the liver is deficient, greater amounts of fatty acids are utilized with the production of more aceto-acetic acid and β-hydroxybutyric acid than can be oxidized by the tissues. These bodies accumulate in the blood and are excreted in the urine. Thus ketosis develops in any of the clinical states of deficient carbohydrate metabolism. The most important of these is diabetes mellitus. A ketosis develops with starvation as the carbohydrate stores are depleted and metabolism of fat predominates. A special type of starvation ketosis develops with prolonged vomiting or diarrhea. An interesting but rare condition described by Van Crevald associated with a ketosis is Von Gierke's disease, in which carbohydrate is stored to an excessive degree in the liver and other organs, but the mobilization is poor. The level of blood sugar is subnormal.

Acetone and diacetic acid each give delicate color reactions with nitroprusside. A faint nitroprusside reaction therefore indicates only a mild ketosis. The ferric chloride reaction is positive only in the presence of a considerable concentration of diacetic acid so a positive test indicates a rather severe ketosis. Tests for β-hydroxybutyric acid are not satisfactory but wita a positive test for diacetic acid and acetone, its demonstration is not necessary for the diagnosis of acidosis.

Detection of Acetone:

Rothera's Test. This test is quite sensitive for acetone and even more so for diacetic acid. In this test about a gram of solid ammonium sulfate is dissolved in about 3 ml. of acidified urine. Three drops of the nitro-prusside solution are added and strong ammonia then placed on top in a layer. A red to purple ring at the point of contact shows the presence of acetone bodies.

Detection of Diacetic Acid:

Gerhardt's Test. To about 3 ml. of urine in a test tube add a few drops of 10 per cent solution of ferric chloride, drop by drop. Centrifugalize the tube to clear the solution and add another drop or two of ferric chloride. If diacetic acid is present a deep purple or port wine color is produced. This same reaction is given when the urine contains elimination products of coal tar derivatives. However, if the color is due to diacetic acid, it will fade on boiling and not reappear when more ferric chloride is added, since diacetic acid is decomposed by heat.

Blood

Blood present in the urine may be in the form of either intact cells, *hematuria*, or just hemoglobin, *hemoglobinuria*. Hematuria may be detected by microscopic examination or chemical test. Its significance will be considered in the discussion of microscopic findings.

Hemoglobin, unaccompanied by red cells, appears in the urine with excessive hemolysis in a number of toxic states, notably: in mushroom poisoning, in poisoning by beans of the species cicia fave (Favism), following severe burns, in the black-water fever of estivo-autumnal malaria, and following reaction from mismatched transfusion. Poisoning by certain chemicals, notably potassium chlorate, pyrogallic acid and carbon monoxide produce hemolysis with presence of hemoglobin in the urine. There are four types of *paroxysmal* hemoglobinuria. The usual type appears with exposure to cold and is associated with syphilis and the development of specific hemolysins as shown by the Donath-Landsteiner test. In a second type (nocturnal paroxysmal hemoglobinuria) the attacks are not associated with chilling and usually come on at night. In the other two types the pigment appears to be myohemoglobin from muscle. In one, the march variety, hemoglobinuria appears with severe muscular exertion; in the other, paralytic hemoglobinuria, paroxysms occur spontaneously accompanied by extreme muscular weakness and cramping.

Detection of Blood:

Benzidine Test. Dissolve a small amount (knife-point full) of benzidine base (Merck's) in 2 ml. glacial acetic acid and add an equal volume of 3 per cent hydrogen peroxide. Add 2 ml. of urine and mix. The appearance of blue color indicates the presence of hemoglobin. Inferior grades of benzidine may require the addition of larger amounts to obtain a positive reaction.

Guaiac Test. Acidify a portion of urine with glacial acetic acid. Add a fresh alcoholic solution of gum guaiac, drop by drop, until there is a slight

turbidity. Add a few ml. of 3 per cent hydrogen peroxide solution. Development of a blue color indicates the presence of hemoglobin.

Pus will produce the blue color but not if the urine is previously boiled.

A thin film of copper left in the tube after testing for sugar will give a positive reaction as will bromides and iodides.

If minute amounts of blood cells are present, the tests are best performed on the sediment from a centrifuged specimen.

Spectroscopic Test for Hemoglobin. A small direct vision instrument with a side mirror and reflecting prism is satisfactory (Fig. 21.3). It should be used with daylight illumination. The width of the slit must be adjusted and the eyepiece focused so that Frauenhofer's lines are distinctly visible.

If the urine is not clear it must be made so by filtration or centrifugation. A few ml. in an ordinary test tube suffices for examination. When this is held before the spectroscope the unmodified solar spectrum will be seen alongside the spectrum modified by the material in the urine. Each hemoglobin derivative may be identified by its characteristic absorption spectrum.

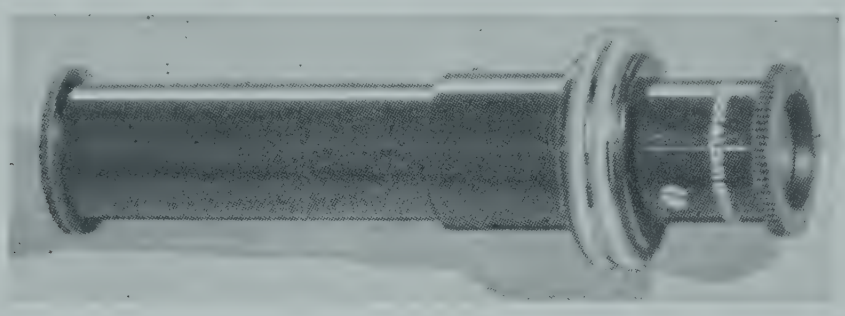


FIG. 21.3. A direct vision spectroscope satisfactory for clinical laboratory use. Courtesy Bausch & Lomb Optical Company.

A spectrum for any of the hemoglobins should be confirmed by a benzidine test.

Porphyrins

The porphyrins are, like bilirubin, iron free pyrrol pigments and, like bilirubin, they are presumably dervied from hemoglobin. Their physiological significance is not known. A very small quantity of one of the porphyrins is excreted normally; quantities of this type sufficient to color the urine may be excreted *with toxic damage to the liver* such as occurs with administration of cincophen, sulphonal, and arsenicals. Another type has been found in the urine with *lead poisoning* and *hemosiderosis*. *Familial idiopathic porphyria* described by Turner occurs as a chronic disease with acute exacerbations; in the latter phase the urine becomes dark red or brown. The excretion of rather large quantities of porphyrin with pellagra has been reported.

Spectroscopic Test for Porphyrin. The spectrum for hematoporphyrin in acid solution is similar to that of oxyhemoglobin. Unlike hemo-

globin, porphyrins give a negative benzidine test; they contain no iron. The porphyrins may be isolated for examination as follows:

To 100 ml. of urine add 10 ml. of 10 per cent sodium hydroxide. The precipitate which settles carries along the porphyrins by adsorption. The precipitate is filtered, washed with water, and then with 95 per cent alcohol. The porphyrin is then dissolved in acid alcohol (10 drops of concentrated HCl in 5 ml. of 95 per cent alcohol). The solution is examined spectroscopically and identification made by the characteristic adsorption spectrum.

Bile

The constituents or derivatives of bile which may appear in the urine are the bile pigments bilirubin and biliverdin; bile acids, chiefly glycocholic acid; urobilin and urobilinogen. The presence of bile pigments and acids together is associated with obstruction to the outflow of bile from the liver. This may be either intra- or extrahepatic. Urinary excretion of bilirubin alone is seen in conditions of excessive hemolysis. Increased amounts of urobilin point to functional incapacity of the liver. Urobilinogen is normally present in the urine in amounts sufficient to give a positive test in dilutions of 1:10 to 1:20. Its absence indicates complete biliary obstruction while increased amounts are associated with excessive blood destruction. For more detailed discussion see Chapter 8, Liver Function Tests.

Detection of Bile Pigment (Bilirubin and Biliverdin):

Gmelin's Test. To a few ml. of urine in a test tube add a small amount of concentrated nitric acid in such a manner that two layers are formed. The formation of a rainbow band of colors at the junction of the layers shows the presence of bilirubin.

Bilirubin Tablet Test. A diazo type test, more sensitive and specific for bilirubin, has been devised. It consists of a tablet containing 0.2 mg. p-nitrobenzene diazonium-p-toluenesulfonate, 100 mg. sulfosalicylic acid, 10 mg. sodium bicarbonate, and 20 mg. boric acid. The procedure is quite simple. Five drops of urine are placed on a small square of test mat. One tablet is placed on the urine and two drops of distilled water are dropped on top of the tablet. The area about the base of the tablet is watched for 30 seconds for the development of a blue or purple color on the mat. Color forming after 30 seconds or on the tablet is ignored. A negative test is no blue or purple color developing on the mat in 30 seconds.

Detection of Bile Acids:

Hay's Test. Upon the surface of cold urine sprinkle a little finely powdered sulphur (flowers of sulphur). If it sinks at once, bile acids are present to the amount of 0.01 per cent or more; if only after gentle agitation, 0.0025 per

cent or more. If it remains floating even after gentle shaking, bile acids are absent.

Thymol preserved urine may give a false positive reaction.

Detection of Urobilin:

Schlesinger's Test. To 2 ml. of urine in a test tube add several (3 or 4) drops of Lugol's solution and an equal quantity of saturated alcoholic solution of zinc acetate. Centrifugalize to throw down the precipitate. Examine the tube while holding it in *direct* sunlight or in the rays from a beam of strong light. A greenish fluorescence indicates the presence of urobilin.

Quantitative Determination of Urobilinogen. *Wallace and Diamond Test.* For discussion, see Chapter 8, Liver Function Tests.

Melanin

The presence in the urine of melanin or its colorless precursor, melanogen, occurs most often in the late stages of melanoma with widespread metastasis but has been reported in a number of unrelated conditions in which the body produces sufficient melanogen to exceed the kidney threshold. Melanogen is readily oxidized to the colored compound melanin, and urine containing appreciable quantities will become dark brown or black upon standing. This reaction may be accelerated by the addition of oxidizing agents such as ferric chloride but the resulting darkening of the urine is neither a sensitive nor a specific test for melanin.

Detection of Melanin. Thormählen Test.

1. To 5 ml. of urine in a test tube add a few drops of sodium nitroprusside solution, then add a few drops of 10 per cent sodium hydroxide solution.
2. A deep ruby color develops which is not specific for melanogen being formed also by acetone and by creatinine.
3. Acidify with glacial acetic acid. The *immediate* development of an azure blue color indicates the presence of melanogen. With acetone alone the color becomes a deeper red, and with creatinine, it becomes yellow, then green and finally blue.

Indican

Indican, skatol and possibly other indol derivatives are formed by the action of bacteria on protein. A positive indican reaction is taken as evidence of putrefaction; it is associated with the clay stools of biliary obstruction, with intestinal obstruction (but not necessarily with constipation), and with the loose, putrid stools of pernicious anemia, sprue and pellagra. It may also be found with empyema or other large suppurative lesions.

Determination of Indican:

1. *Obermayer's Test.*

Procedure. Transfer about 3 ml. of urine to a test tube. Add an equal volume of Obermayer's reagent and about 1 ml. of chloroform. Stopper the tube and mix by repeated inversion, and allow the chloroform to settle to the bottom of the tube. A blue color indicates the presence of indican. A reddish coloration indicates skatol or some other indol derivative. A red coloration is also given by iodides, but this will fade if a few crystals of sodium thiosulphate be dropped in and gently agitated. The urine should not be preserved with formaldehyde as this interferes with the test. Hexamethylenamine also interferes. Bile pigments likewise interfere but may be removed from the urine by previous treatment with calcium or barium hydroxide or lead acetate.

Alcaptonuria

Alcaptonuria is one of the inborn errors of metabolism in which there appears to be a failure to completely oxidize tyrosine and phenylalanine with the resulting excretion in the urine of alcapton or homogentisic acid. The condition is inherently benign, has a hereditary tendency and occurs more frequently in males than in females. It is occasionally associated in ochronosis with the excretion of melanin.

Detection of Homogentisic Acid:

1. *Alkali Test.* The addition of 10 per cent sodium hydroxide to alcaptonuric urine produces a brown color in 1 to 2 minutes.
2. *Benedict's Qualitative Test.* The technic is the same as that for sugar in the urine. The reduction is atypical ending in a brown to black color.
3. *Ferric Chloride Test.* Upon the addition of dilute ferric chloride solution, a deep blue color appears for a moment until oxidation is complete.

MICROSCOPIC EXAMINATION

Microscopic examination is one of the most important procedures in urinalysis. The gross appearance of a urine specimen can not be used as an indication for or against such examination. Clear specimens with little or no visible sediment may reveal important findings while cloudy urine with heavy sediment may contain nothing of clinical significance. All specimens should be examined as soon as possible after being voided, certainly within six hours. If greater delay is necessary, the formed elements are best preserved by formalin.

Preparation of Specimens

The objects that are found by microscopic examination are suspended in the urine and should be concentrated by adequate centrifugation.

After centrifugalizing, pour off the supernatant fluid leaving the sediment and one or two drops of urine. Shake the tube to break up the sediment and place on a slide a drop sufficiently large to fill the space beneath a cover slip without floating the slip. Cover slip preparations should be used routinely as they reduce motility of objects in the field and prevent soiling of the objective when high power magnification is used. Also, only by this technic can essentially the same amount of sediment be examined each time, thus permitting a rough but satisfactory day to day comparison of the numbers of formed elements present. Some workers add a drop of 25 per cent acetic acid to bring out cell nuclei, especially if there is difficulty in distinguishing between red and white cells.

If examination for minute details of cells and bacteria is done, it is better to use a stained preparation. This is prepared by smearing a loopful of sediment on a slide and staining in the usual manner with Gram's, acid fast, Wright's or simple methylene blue stain, as indicated.

Method of Examination

Examine the sediment first with the low power (16 mm.) lens. The light of the microscope must be adjusted (reduced) so that hyaline objects and those with little color may be seen well. This is done by first centering the full maximum light on the object and then closing the substage diaphragm about three-fourths. It may be necessary to use the high dry lens (4 mm.) for higher magnification to identify some particular object.

Place one corner of the preparation in position for examination, and by means of the mechanical stage move the slide from end to end, dropping down the width of a field each time until the entire specimen is examined.

Barring lack of familiarity with the content of urinary sediment, error in examination is usually due to one or more of the following factors:

1. Careless transfer of the sediment, i.e., the part examined not being representative.
2. Use of too much light. This is the most common error of beginners. Unstained objects such as casts are completely invisible in strong light.
3. Use of too high magnification. Constant use of high power magnification invariably results either in inadequate coverage of the material to be examined or great waste of the examiner's time.

4. Drying of the sediment. If the preparation is allowed to stand too long before being examined, salts precipitate and obscure any objects of importance which may be present.
5. Dirty equipment. Dust particles on the surface of the cover slip, slide or even the substage condenser may be erroneously considered a part of the sediment.

Findings are reported as the average number of objects seen per high or low power field, or in absolute numbers where the Addis technic is employed. Statement should also be made as to whether the urine specimen was voided or catheterized and whether examined centrifuged or uncentrifuged.

The Addis Count. The number of cells and casts in the urine sediment may be determined roughly as follows. Obtain a twelve hour specimen of night urine after fluid restriction and measure the volume. Concentrate 10 ml. of this in the centrifuge and with a pipette remove 9 ml. of the supernatant fluid. Mix the sediment in the 1 ml. of fluid that remains and mount a drop of the mixture in the counting chamber. Casts, red cells, and white cells can be counted throughout the entire chamber, the number computed and expressed in cells or casts per 1 ml. of urine. This, multiplied by the amount excreted, gives the number in the twelve hour specimen.

Example: If 18 casts are counted in the entire ruled area of the counting chamber (9 sq. mm.) then.

$$\frac{10}{9} \times \frac{18}{1} = 20 \text{ casts in 1 cu. mm. of concentrated urine.}$$

The urine was concentrated ten times by centrifuging, so,

$$20 \div 10 = 2 \text{ casts per cu. mm. of urine.}$$

$2 \times 1000 = 2000$ casts per cu. cm. If the total volume is 400 ml. then $2000 \times 400 = 800{,}000$ casts in a 12 hour specimen of urine.

The number of bacteria, pus cells, and other cellular bodies can be computed in a similar manner.

Diagnosis of Microscopic Objects

Crystals. Crystals are usually present in the urinary sediment and have relatively little significance. Generally their presence indicates that the urine has stood for a considerable time since being passed. In alkaline urines triple phosphate and ammonium urate crystals are usually observed. The phosphates frequently appear in the large prismatic form. In acid urines calcium oxalate, sodium acid urate and uric acid crystals are commonly seen. Both urate and phosphate salts are fre-

quently present in a non-crystalline, amorphous form. The various crystalline structures are shown in Plate 21.I. Tyrosine (sheaves of needles) and leucine (spheroids) may be present in urine with acute yellow atrophy of the liver. Cholesterol plates with their characteristic missing corner may be observed in nephritic conditions. Cystine crystals, hexagonal with well defined edges, may be present in cystinuria, one of the inborn errors of metabolism.

Crystalline forms of the various sulfonamide drugs are frequently present in the urine. The shapes of the crystals are sufficiently characteristic to allow identification of the sulfonamide drug administered (see Figs. 21.4, 21.5, 21.6). Most of the crystals observed are of the acetylated compounds, with the exception of sulfadiazine which is relatively insoluble in the free form.

Red Blood Cells. The sediment from a *normal* twelve-hour specimen of urine contains about 67,000 red cells (Addis count) which represents only an occasional cell per low power field as usually observed. Increased numbers may be seen in many varied pathologic states. In the female, the uterus and vagina must be eliminated as a possible source (examine catheterized specimen).

The presence of abnormal numbers of red blood cells in the urine indicates either a bleeding lesion somewhere in the genitourinary tract or damage to the glomerular filter, and frequently necessitates cystoscopic examination to determine the exact source. Blood alone, in large quantities suggests a tumor of the bladder or kidney, or if associated with renal colic, calculi are likely. These may be in the kidney, ureter or bladder. Renal infarction usually produces a transient hematuria which may escape detection. Hematuria of unilateral renal origin is the most common early evidence of tuberculosis of the kidney.

Acute glomerulonephritis is associated so commonly with the presence of red cells in the urine that it is often called hemorrhagic nephritis. As the condition becomes inactive or passes into the subacute or nephrotic stage, the hematuria disappears. Blood in the urine is uncommon in chronic glomerulonephritis unless accompanied by heart failure.

Malignant nephrosclerosis frequently causes red cells in the urine but in arteriosclerosis with benign hypertension the finding is usually not a part of the picture.

Hematuria, following trauma to the abdomen or pelvis, should always be sought for and if present, indicates rupture at some point in the urinary tract. Frequently the quantity of blood present is large and

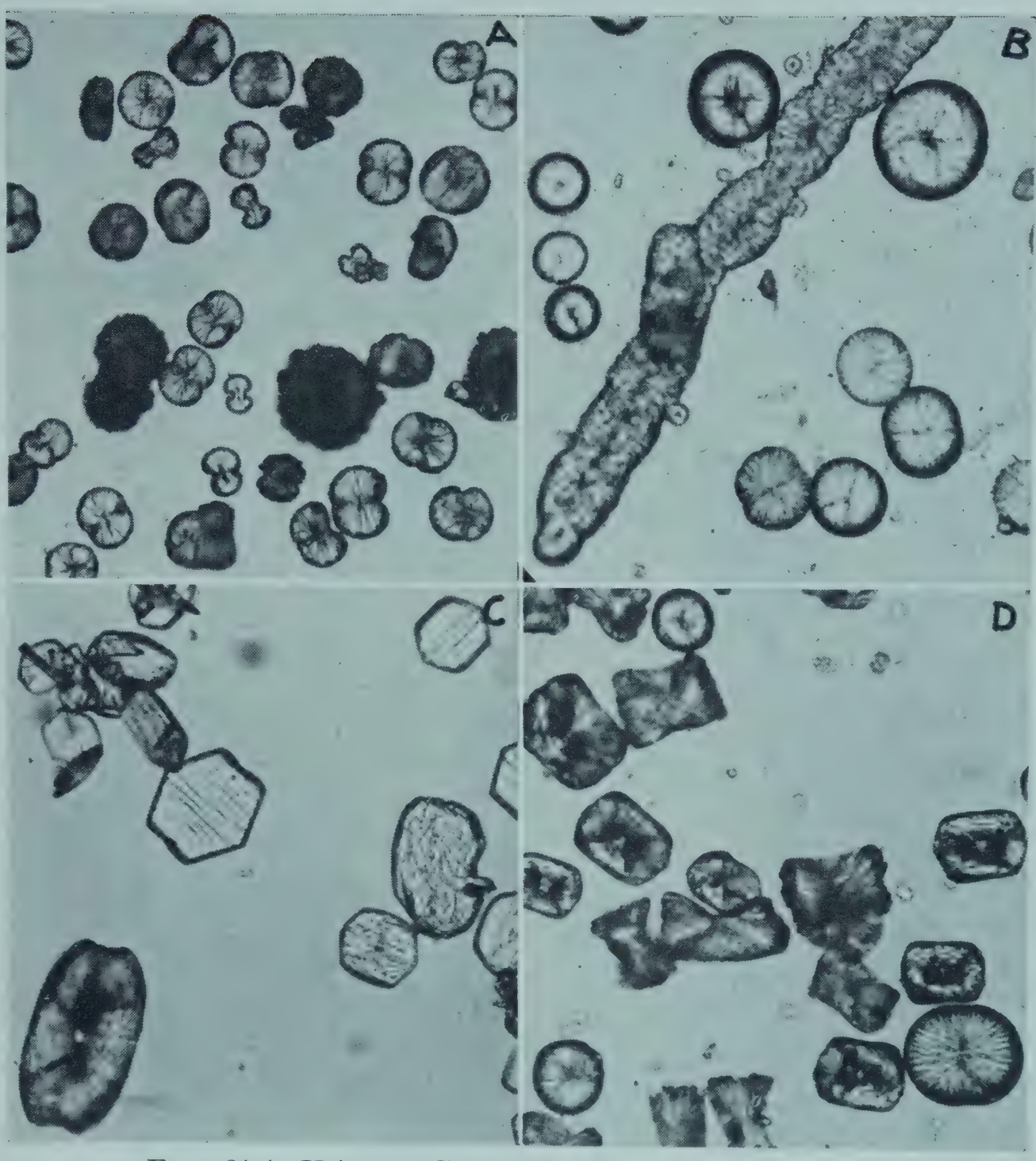


FIG. 21.4. Urinary Crystals of Acetylsulfathiazole

A. Dumb-bells or "shocks of wheat with central binding"; the crystals start as small slender sheaves and grow in such a way that the two half-circle parts finally close up to form a rosette (0.004 by 0.06 to 0.06 by 0.08 mm.).

B. Rosettes (0.04 to 0.1 mm.), the large rod-like structure, consisting of acetylsulfathiazole, may represent a "crystalline cast." On microscopic examination both crystal forms (A and B) appear amber green and show pronounced radial striation.

C. Hexagonal Platelets (0.04 by 0.06 to 0.15 by 0.2 mm.). Sometimes very thin glass-clear, more often thick amber green with a distinct envelope-like pattern. Some of the crystals may reach considerable size and two opposite edges may appear indented resulting in shapes as shown in the right lower corner of the photograph.

D. Demonstrates the simultaneous presence in the same urinary specimen of the forms outlined in A, B and C, a rather frequent observation (Enlargement × 125.) Courtesy of Dr. David Lehr and Dr. William Antopol.

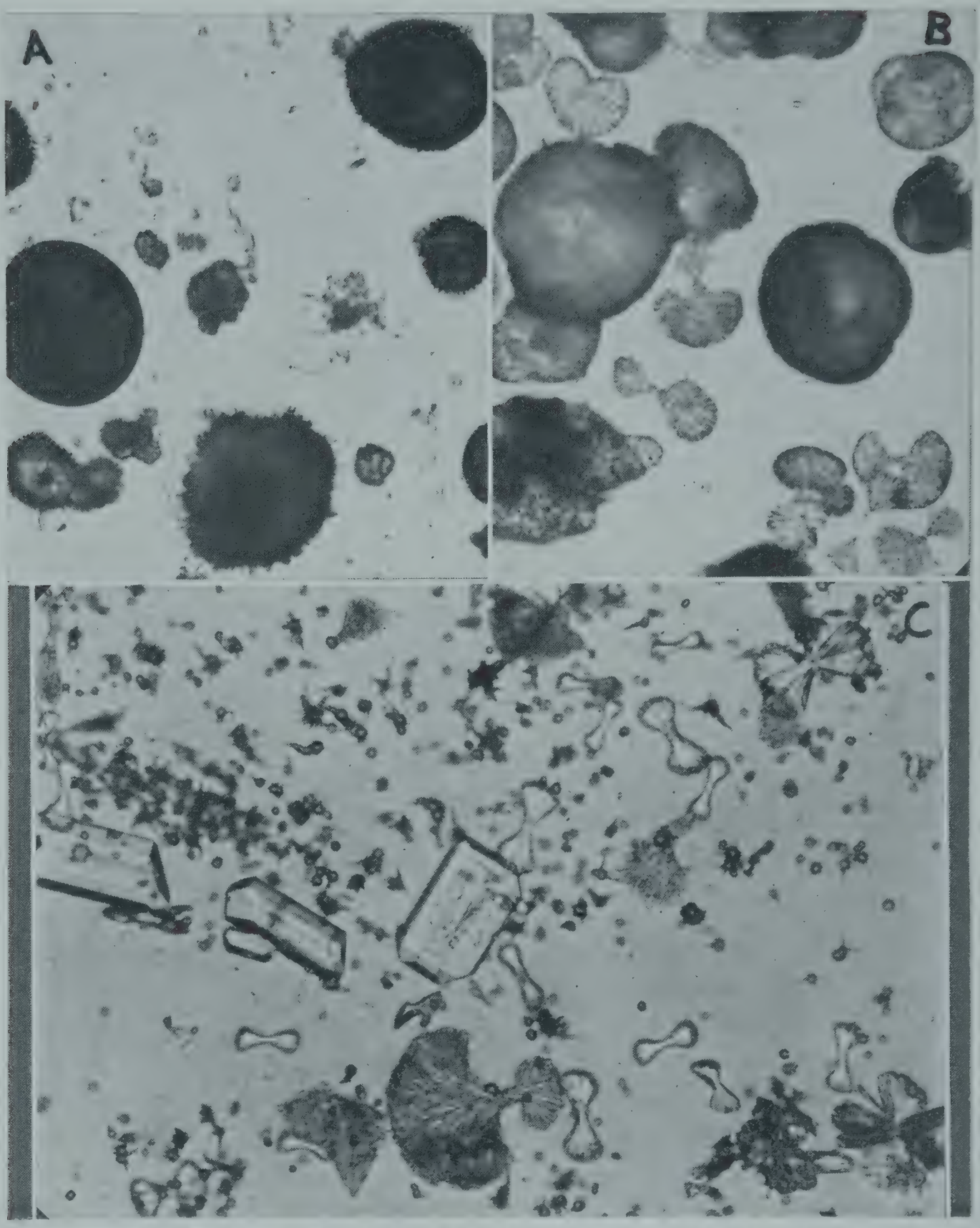


FIG. 21.5. Urinary Crystals of Sulfadiazine and Acetylsulfadiazine

A. Dark, muddy green globules, sometimes covered with needle-like processes (chestnut burr forms), consisting almost entirely of free sulfadiazine (0.03 to 0.16 mm.).

B. Among "globules" containing free sulfadiazine, "shocks of wheat with excentric binding" and "shell" forms, consisting mainly of acetylsulfadiazine. Like the corresponding sulfathiazole crystals they are amber green and have a conspicuous radial striation (0.02 to 0.14 mm.).

C. Urinary sediment of a patient who had been switched from sulfathiazole to sulfadiazine treatment 24 hours before voiding the urine specimen containing these crystals. A perfect "shock of wheat with excentric binding" (acetylsulfadiazine) can be seen among symmetrical dumb-bells (acetylsulfathiazole). Also present are typical phosphate and monourate crystals. (Enlargement × 156.) Courtesy of Dr. David Lehr and Dr. William Antopol.

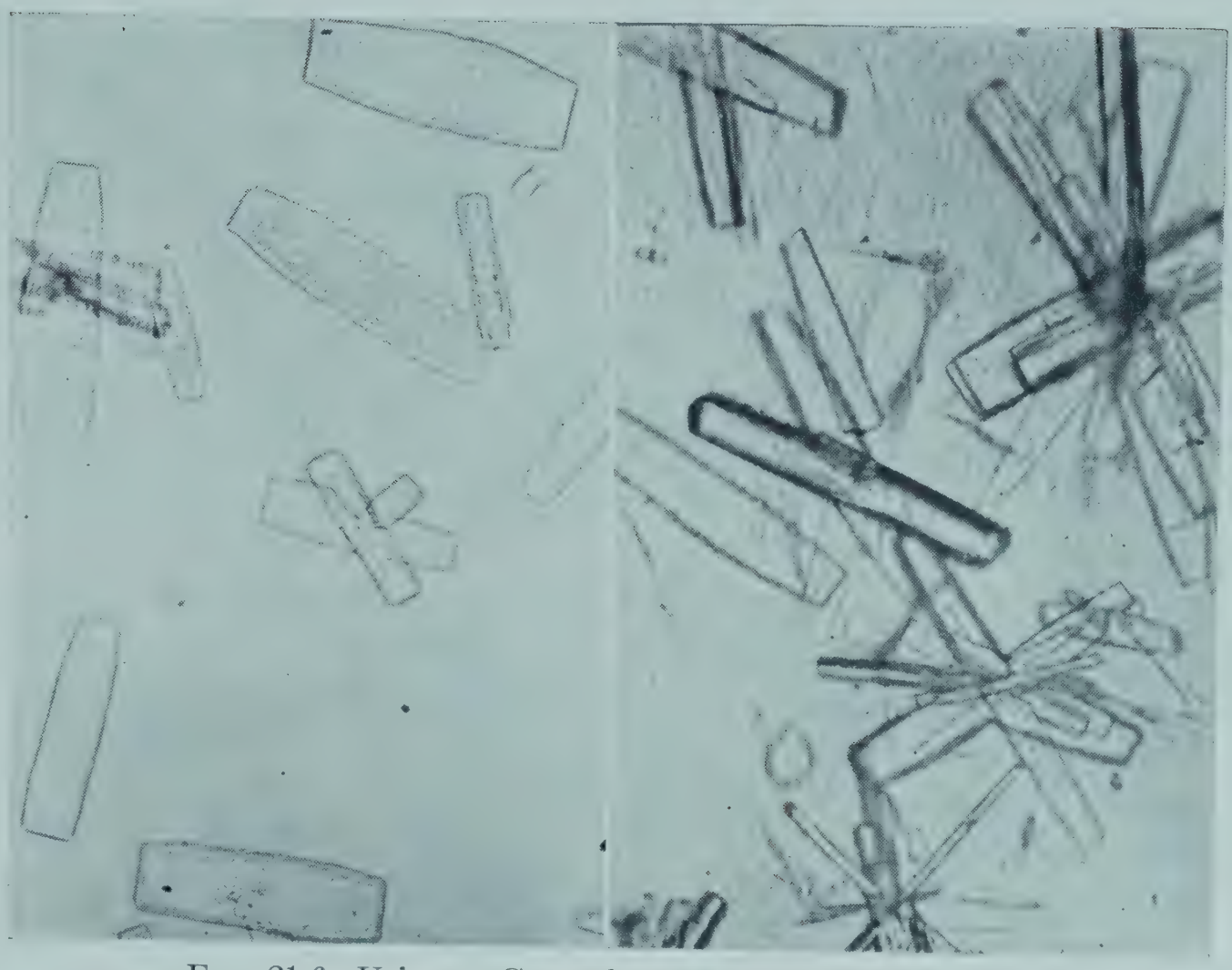


FIG. 21.6. Urinary Crystals of Acetylsulfaguanidine

Simple rectangular oblong plates with slight bulging in the long axis. Markedly varying in size (0.02 by 0.09 by 0.24 mm.), these plates are either glass-clear or have a fine mesh-like pattern, which makes them appear like isolated plant cells or slender tape worm proglottides. They tend to conglomerate, forming cross- or star-like structures. Repeatedly slender sheaves of needle crystals were seen interspersed among the plates (center in the upper part of the right photograph). (Enlargement × 138). Courtesy of Dr. David Lehr and Dr. William Antopol.

accompanied by clots. The two most common sites of trauma are the kidneys and the membranous urethra.

The kidneys and bladder are frequently sources of hemorrhage in purpura of both the idiopathic thrombopenic and the allergic types. In these conditions the hematuria may be associated with purpuric manifestations elsewhere, or may occur alone. One patient has been observed to bleed to death from her kidneys in allergic purpura. Similar hemorrhage from the kidneys is not uncommon in hemorrhagic disease of the newborn.

During the administration of sulfonamides, particularly sulfadiazine, the urine should be watched carefully for the appearance of red blood cells. Their presence indicates crystallization of the drug in the kidneys with tubular damage, and forewarns the necessity of either discontinuing the drug or increasing urinary output to prevent renal precipitation. Routine microscopic examination of the urinary sediment for red blood

cells every three days during sulfonamide administration has been recommended.

Red blood cells accompanied by pus cells is a characteristic urinary finding in cystitis, pyelonephritis, and other suppurative conditions of the urinary tract.

Red blood cells (Plate 21.I) are usually somewhat altered by the urine. They may be swollen or faded or crenated and appear greenish instead of red. They may be differentiated from other objects by mixing a little 25 per cent acetic acid with the material on the slide which hemolyzes the blood cells but accentuates the other cells, such as pus cells and epithelial cells.

Pus Cells. A few leukocytes are found in acute glomerulonephritis, along with red cells and epithelium. Otherwise pus in the urine indicates a suppurative process somewhere in the genito-urinary tract, as in pyelonephritis, cystitis, or urethritis. Normally in the female leukocytes arise from the genital tract, and have a limited significance except in catheterized specimens of urine.

Pus cells (Plate 21.I) are recognized by their characteristic granular cytoplasm and nuclei which can be easily identified with proper adjustment of the light. If in doubt 25 per cent acetic acid can be added to bring out the nuclei better or a suitable preparation may be made and stained. The presence of 80,000 to 100,000 pus cells per cubic millimeter adds 0.1 per cent of albumin.

Epithelial Cells. Squamous epithelium from the bladder, urethra, and particularly the vagina, is found in normal urine sediment. A few round epithelial cells are observed frequently in normal urine. They are thought to come from the renal tubules, and they are much increased in hemorrhagic and degenerative nephritis, and with chronic passive congestion from heart failure. Caudate epithelial cells from the renal pelvis may also be present.

Epithelial cells are shown in Fig. 21.7. Squamous epithelial cells are easily recognized as large flat cells with rather small nuclei. They may be rolled up so that they resemble cigars, or they may even be mistaken for casts. The cells from the renal tubules are slightly larger than leukocytes and have a single round nucleus.

Casts. Casts are passed in very small numbers by normal individuals (about 1000 per 12-hour specimen, Addis count) but increased numbers are seen in various stages of renal irritation.

In acute glomerulonephritis large numbers of casts are passed, not only during the initial stage, but throughout the latter stages of activity.

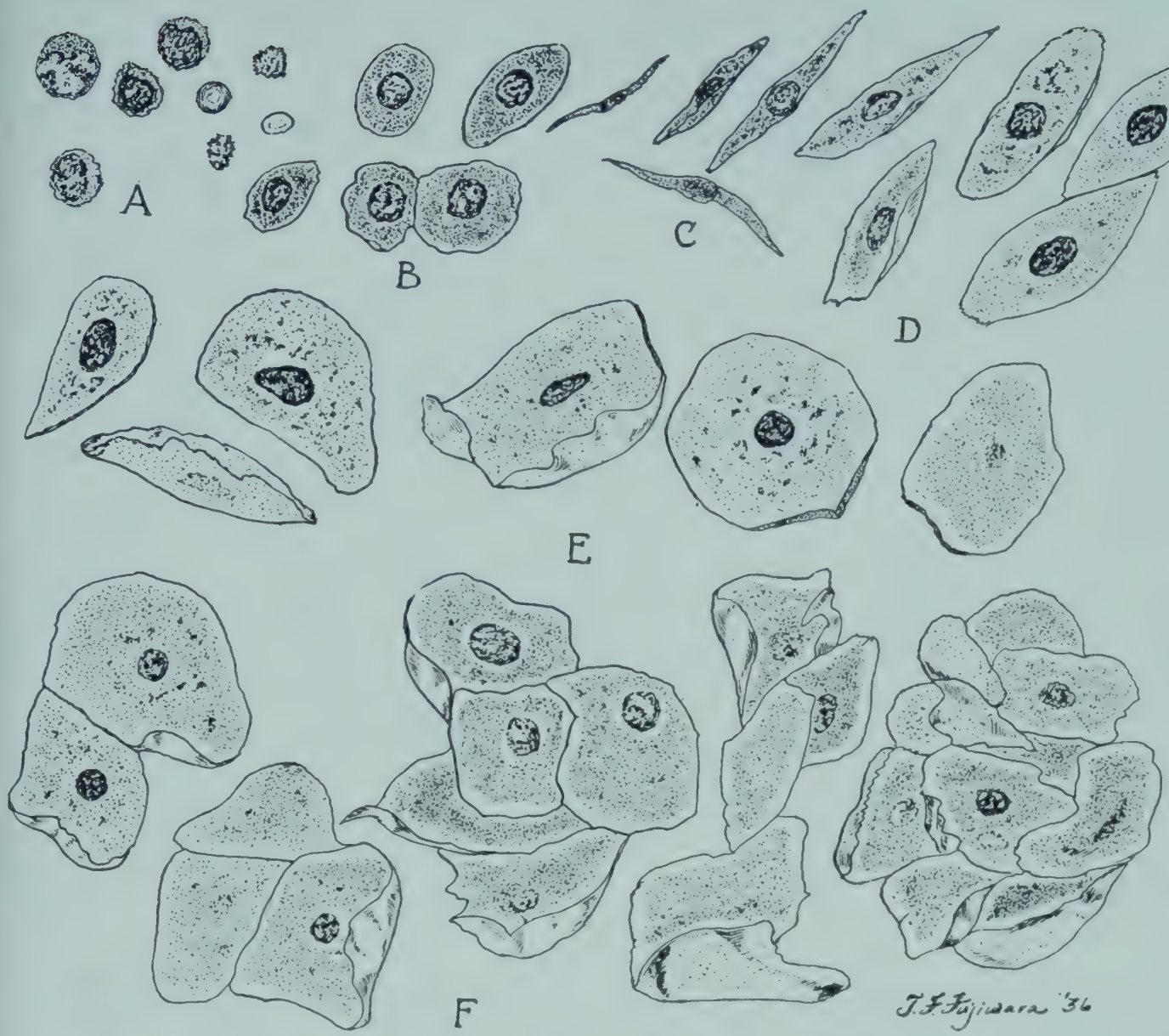


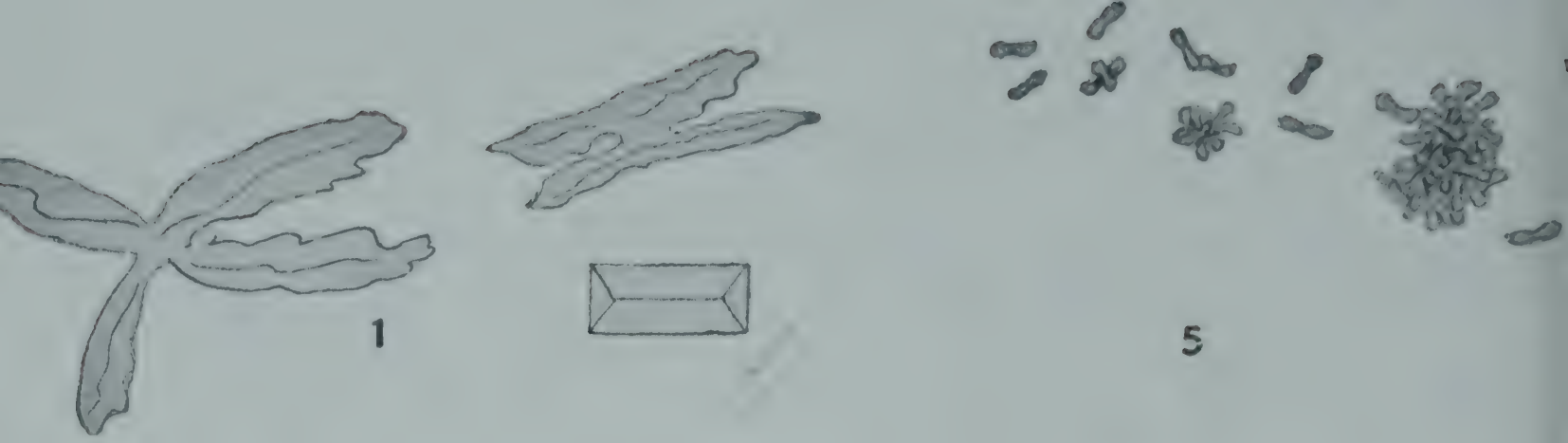
FIG. 21.7. Types of cells found in urine. A. Leukocytes and pus cells. B. Renal epithelial cells. C. Caudate epithelial cells from kidney pelvis. D. Cells from urethra and bladder. E and F. Single and clumped squamous epithelial cells from bladder and urethra.

Because of the accompanying hematuria, some of the casts contain red cells or orange colored red cell debris. All of the other types of casts may also be observed in this condition. (See Plate 21.I.)

In degenerative nephritis the red cell excretion is approximately normal; blood casts are not observed, but granular, fatty, waxy, and hyaline casts are passed in large numbers. It must be borne in mind that a degenerative type of nephritis occurs in the toxemias of pregnancy, as well as in various other toxic conditions, and that the urinary sediments in these various states are qualitatively similar.

Nephrosclerosis tends to produce relatively little sediment, but a few hyaline and granular casts may be seen. With heart failure, which frequently supervenes in this disease, the casts become more numerous.

Suppurative kidney diseases produce relatively few casts and these are usually pus casts.

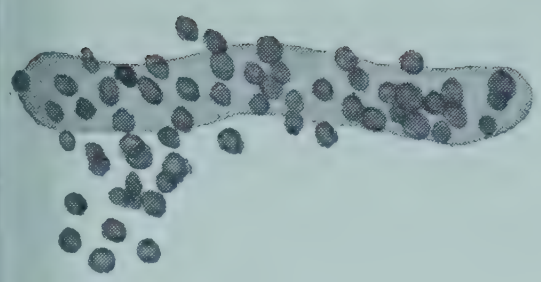




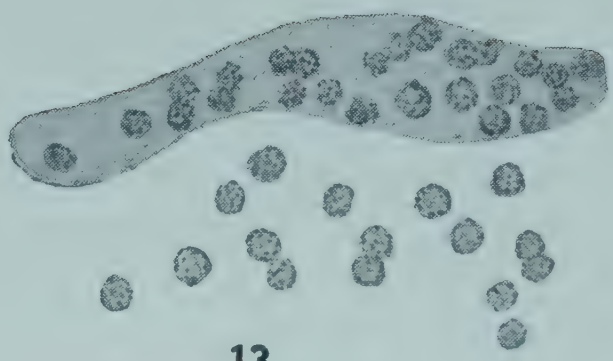
Nos. 1 and 3. Triple phosphate crystals.
No. 2. Calcium phosphate crystals.
No. 4. Uric acid crystals.
No. 5. Calcium carbonate crystals.
No. 6. Ammonium urate crystals.
No. 7. Leucine spheres.
No. 8. Tyrosine needles.
No. 9. Calcium oxalate crystals.
No. 10. Sodium urate crystals.
No. 11. Cystine crystals.

PLATE 21.I

Microscopic Examination of the Urine



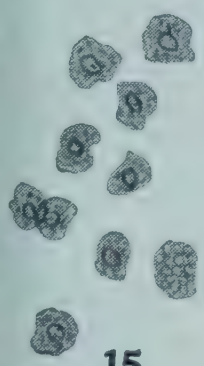
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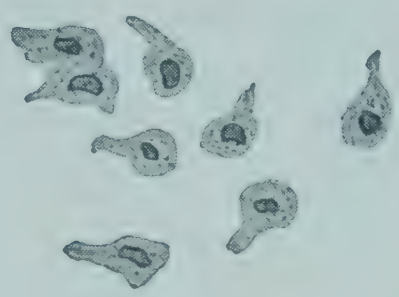
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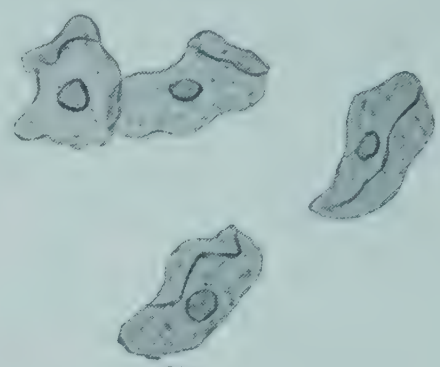
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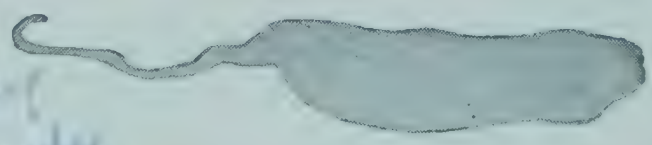
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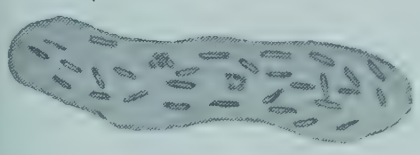
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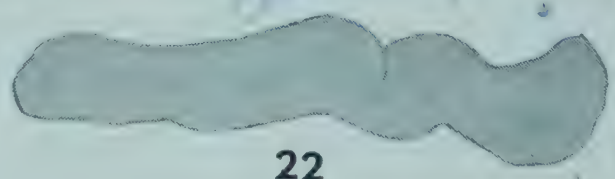
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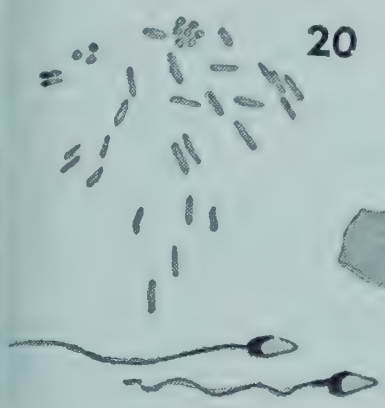
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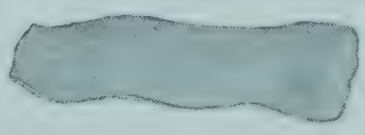
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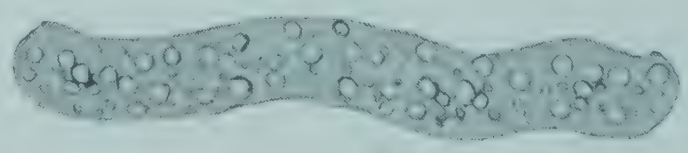
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No. 12. Blood cast.
No. 13. Pus cells (Leukocytes).
No. 14. Epithelial cell cast.
No. 15, 16, 17. Epithelial cells.
No. 18. Mucus threads.
No. 19. Cylindroid.
No. 20. Bacterial cast.
No. 21. Granular cast.
No. 22. Hyalin cast.
No. 23. Spermatozoa.
No. 24. Waxy cast.
No. 25. Blood cast.

PLATE 21.I (*continued*)

Microscopic Examination of the Urine

Amyloidosis of the kidneys produces no characteristic sediment. Waxy or *amyloid* casts do not contain true amyloid.

The very broad, short, renal failure casts of Addis occur in advanced stages of various types of nephritis with contracted kidneys.

Casts (Plates 21.I and 21.II) are of several varieties. They are casts of uriniferous tubules and therefore vary in size and shape according to that of the different tubules in which they are formed. As the casts are forced out of the tubules, they are often broken so that some are short pieces while others are very long but most are of intermediate lengths. Some are two or three times as thick as others. The basis of all casts is a hyaline-like material and when they consist of this material only, they are called hyaline casts. The hyaline material appears to be composed of a mixture of a mucoid substance secreted by the tubular epithelium, and coagulable globulin, which in relatively small quantities passes the glomerular filter with serum albumin. In some instances of kidney disease, there is loss of intercellular cement of the tubular epithelium, and some of the epithelial cells become detached. When these either adhere to or become incorporated in the hyaline substance, it is known as an *epithelial cast.* These cells are usually swollen and tinged with a yellowish or brownish color. An epithelial cell cast, therefore, is a hyaline cast in which epithelial cells are embedded.

If the damaged epithelial cells are not carried out with casts as such, they continue to degenerate, breaking up into coarse granules. These granules adhere to casts that form in the tubules and such casts are now called *coarse granular casts.* The granules vary from a light yellow to dark brown in color. A coarse granular cast, then, is a hyaline cast with coarse granules adhering to or embedded in it.

If the granules are not carried out in cast form while they are coarse, they continue to break up into even finer granules and these become embedded in the casts. Such casts are called *fine granular casts.*

In other diseases of the kidney the degeneration of the tubular epithelium is a fatty type instead of granular degeneration, and casts formed in such tubules then have fat droplets on or in them. Such casts are called *fatty casts.* A fatty cast is a hyaline cast with fat droplets on the surface or within the cast. In other diseases the red blood cells may pass into tubules where casts are forming and adhere to them. The red cells are usually not intact but only red or orange colored debris is seen. Such casts are called *red blood cell casts.* Likewise, pus cells may be attached to casts forming *pus cell casts.*

There are often various combinations of the above different varieties

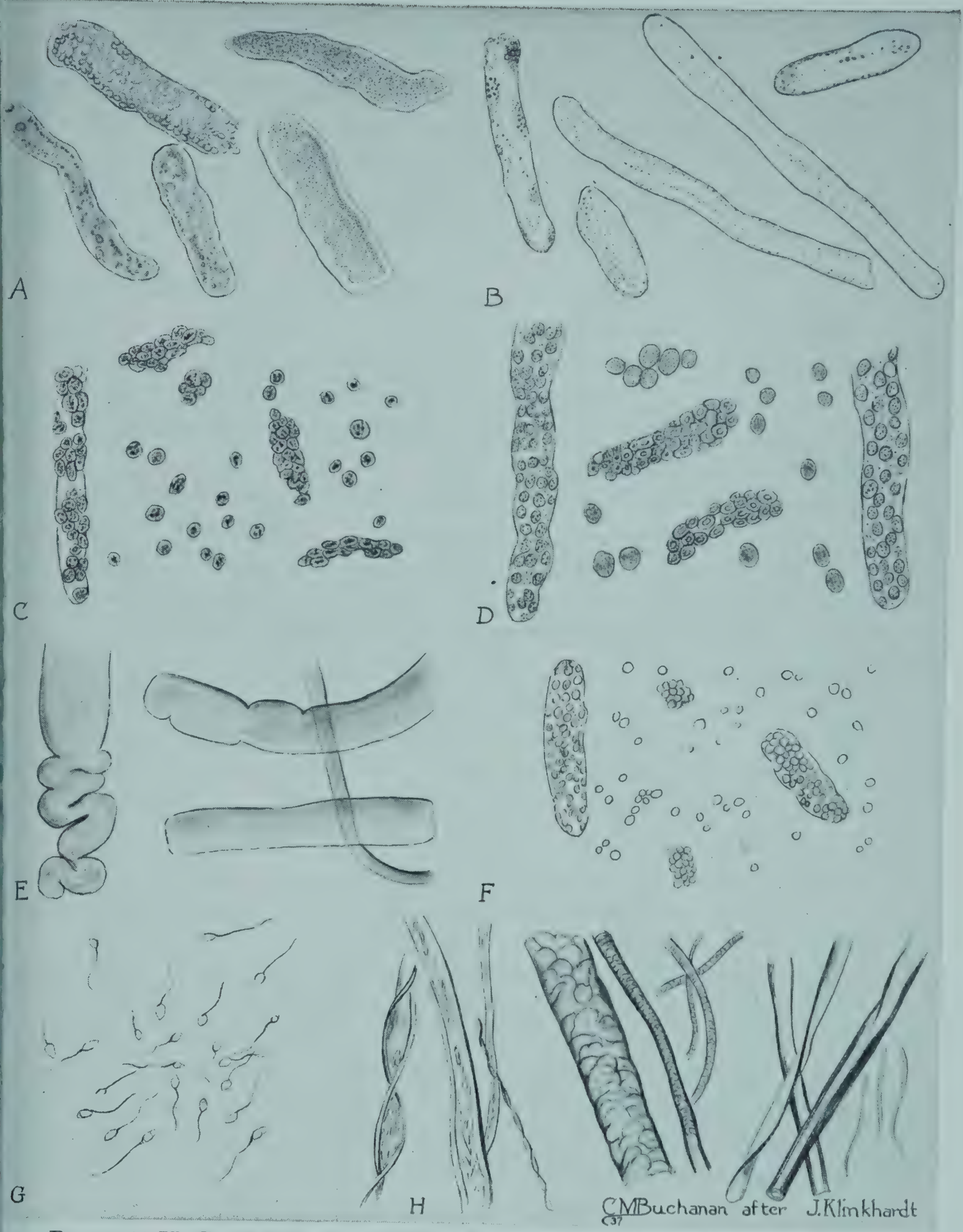


PLATE 21.II. Casts and other findings in urine. *A*, fine and coarse granular casts. *B*, hyaline casts. *C*, leukocyte casts with scattered leukocytes. *D*, epithelial casts with scattered renal epithelial cells. *E*, waxy and colloid casts. *F*, blood casts (erythrocyte casts) and scattered red cells. *G*, spermatozoa. *H*, cloth fibers in urine. From left to right, cotton, wool and silk. (Original drawing adapted from Atlas of Urinary Sediments by Hermann Rieder, translated by A. Sheridan Delepine.)

of casts. For instance, a single cast may contain epithelial cells, coarse granules, fine granules, and fat droplets. Also, one end of a cast may be hyaline with the other end loaded with granules.

Rarely casts consist of a highly refractive waxy material of light yellowish color instead of the usual hyaline material. Such casts are called *waxy casts*.

Cylindroids, mucous threads, vegetable fibers or masses of amorphous phosphates may be mistaken for casts.

Cylindroids resemble hyaline casts, but have one end tapering out to a thread. Some of these may have only a fine thread projecting from an end; consequently the distinction between casts and cylindroids is difficult in some instances.

Mucous threads are long, very slender and transparent, and tend to be curled or entwined into various patterns. They may be seen in various irritations or inflammations of the urinary tract. They should not be confused with cylindroids and certainly not with vegetable fibers which are more opaque and have well defined edges.

Spermatozoa. Spermatozoa may be present in the urine of men after epileptic convulsions, nocturnal emissions, and in spermatorrhea; also in the urine of both sexes after coitus. They are detected best in a moist preparation using subdued light. (See Plates 21.I and 21.II.) In fresh alkaline specimens, motility may be preserved. They also show well in stained specimens.

ANIMAL PARASITES

Endamoeba histolytica has been reported in the urine.

Trichomonas. Either *Trichomonas hominis* (see Chapter 14, section on protozoa), or *Trichomonas vaginalis* may be found in the urine and may or may not be associated with irritation or mild inflammation of the bladder. *T. hominis* may become established in the urinary bladder. *T. vaginalis*, when found in the urine, may come from the bladder or from the vagina by contamination of the urine with vaginal secretion. In specimens from males *T. vaginalis* infections may be chronic in the urethra, prostate, or seminal vesicles.

Schistosoma haematobium (see Chapter 14, Plate 14.V), the blood fluke which inhabits the veins in the wall of the urinary bladder, deposits its eggs in the capillaries of the mucosa. Minute abscesses develop about the eggs producing ulceration and polyposis. The eggs are found in the urine accompanied by blood and pus. This condition is endemic only in Africa but should be suspected in individuals who have lived in

endemic areas. A history of foreign service with the finding of blood and pus in the urine warrants a careful search for these ova.

Ova of the pin worm (*Enterobius vermicularis*) have been found in urine. This may be due to contamination in voided specimens from females but in one instance infection of the bladder was verified on catheterized urine.

ARTIFACTS AND EXTRANEOUS SUBSTANCES

Frequently objects find their way into urine during the collection, transportation or preparation of the specimen or become mixed with the sediment after it is put on the slide. The more common ones should be recognized so they may not be confused with structures of pathologic importance.

Yeast cells are frequently seen. These are generally oval in shape and are about the size of a pus cell although there may be considerable variation. Budding is especially characteristic. They show an intense Gram positive reaction when stained.

Starch granules vary considerably in size and shape; those from corn are irregular, often with jagged edges, while those from potato are quite similar in appearance to yeast cells, even showing small protuberances as in budding yeast forms. The granules are slightly refractile and turn blue-black on contact with weak iodine solutions.

Oil droplets are often present particularly in urine from women who have been taking mineral oil (contamination). These are spherical, vary in size and show concentric rings of light refraction upon focusing up and down with the fine adjustment. *Air bubbles* when small have similar shape but show a definite outline without refractive rings.

Fibers are commonly seen, the most frequent being cotton although wool and silk may be present. These are shown in Plate 21.II.

Pollen granules show varied and bizarre forms, each characteristic of the plant by which it is formed. These gain access to the urine from flowers in the sick room or from the atmosphere in seasons of heavy pollination.

Scratches on the slide may be a source of confusion, hence slides should be discarded when they become too badly etched. Dirt or dust on the preparation or in the optical system has already been cited as a source of error.

Examination of the urinary sediment is important not only for red blood cells and casts, signs of renal irritation, but also for crystals. Specific crystals of the sulfa drugs are easily identified in acid urine. In

addition, the crystals of oxalate, if present, indicate a possible toxic state due to oxalic acid or ethylene glycol. The presence of tyrosine, leucine, and cystine crystals indicates tissue destruction and they are most commonly seen in yellow atrophy of the liver due to a variety of toxic substances (see Plate 21.I).

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CHAPTER 22

SALIVA, SPUTUM, AND BRONCHIAL ASPIRATES

By Seward E. Miller

Saliva

GENERAL CONSIDERATIONS

The mixed product of secretions from the parotid gland (serous) and the submaxillary and sublingual glands (mixed serous and mucus) is saliva. It is a thin, clear, water, slightly viscid fluid, normally having a pH between 6.2 and 7.2 with an average of 6.75. Among its more important functions are those of moistening and lubricating the mouth and lips, some cleansing, and perhaps some mild bactericidal effects. In periods of dehydration the lack of secretion causes dryness and a sense of thirst develops. Saliva mixes with food in the mouth during mastication, helping develop a suitable consistency for swallowing and the enzyme ptyalin—the salivary amylase—digests starch to maltose. Although time in the mouth is rather short the enzyme action may continue in the stomach for some time. There appears to be considerable variation in the amounts of saliva produced both resting and upon stimulation of the salivary glands.

INTERPRETATION

Examination of saliva is rather infrequently performed in most clinical laboratories because of either the lack of confirmed clinical significance of most tests or simpler and more precise methods of obtaining similar information. However, loss of buffering action and increased acidity of saliva may play a significant role in dental caries, so volume and particularly pH may be quite significant in some cases of dental caries. The first stage of carbohydrate metabolism starts in the mouth with the conversion of carbohydrates to maltose and dextrin. Determination of salivary amylase may be of considerable significance in studying cases with deficient carbohydrate metabolism, particularly in older individuals where decreased salivary amylase production occurs frequently.

In addition, the work of Richardson suggests that the female salivary glands are selective in their excretion of blood borne hormones, retaining female-associated hormones but allowing free passage of male-associated

hormones. This work may prove of value in pregnancy tests and of assistance in determing the sex of the developing fetus. For further discussion refer to Chapter 16, The Assay of Hormones.

Sputum

GENERAL CONSIDERATIONS

The mucosal surface of the respiratory tract is kept rather constantly moist and sticky by a thin mucus secretion arising from both mucus glands and many goblet cells. It functions as part of the protective mechanisms to help entrap particulate matter and bacteria that are present in most air inhaled into the respiratory passages. Such air, upon reaching the alveoli, is practically free of particulate matter and sterile. Possibly the mucus is slightly bactericidal; if so, very weakly. At any rate the cilia which are present down to the smaller bronchioles are constantly sweeping this mucus, accumulating particulate matter and bacteria upward at a rate of as much as 1 cm. per minute. The amount of mucus normally is rather small but large quantities can be produced by local irritation, inflammatory process, or by reflex action. The accumulation of any appreciable amounts of mucus in the bronchi initiates the cough reflex and the material thus produced from the bronchi is sputum.

Normally, the volume of mucus secretion derived from the respiratory tract is too small to excite cough and expectoration but any irritation of the mucus membrane will increase the amount and thus incite coughing in an attempt to rid the lung of the accumulated material. Sputum, therefore, may be regarded as a pathologic secretion produced in the bronchioles, bronchi, trachea, or from a combination of these, or from material formed elsewhere accumulating in the respiratory tract, and is largely composed of mucus or inflammatory exudates.

In order that accumulated material may be coughed up, there must be a communication with the bronchus and the material must be sufficiently fluid for expectoration. Hence, there may be an accumulation of material in the lungs with the absence of sputum. It may consist essentially of a serous transudate in pulmonary edema, or be derived from the pleural or peritoneal cavities, the esophagus, tracheo-bronchial glands, cysts of the liver, etc., by rupture into the trachea, bronchi or lungs.

Bloody sputum may be due to tuberculosis, passive congestion of the lungs, infarction, hemorrhagic inflammation involving the alveoli,

bronchi or trachea, ulceration of the bronchi or trachea or from blood vessels or various other communicating sources.

In the presence of catarrhal states or infections of the nose or throat, a certain amount of mucoid material may accumulate in the bronchi at night and be dislodged by coughing. However, strictly speaking, such secretions are not sputum.

Sputum shows considerable variation, both quantitative and qualitative, in different diseases and may change with the progress of the disease. Therefore, study of the sputum may yield valuable data bearing on the disease in question and repeated examinations are particularly valuable. This is especially important in searching for tubercle bacilli.

Any condition in which sputum is expectorated or where there is respiratory irritation, and the presence of any disease of the lungs and upper respiratory tract, are indications for examination of sputum.

COLLECTION OF SPECIMENS

For purposes of measuring the total output of sputum per day, a 24-hour sample is required. This can be collected in a clean wide-mouthed bottle of sufficient size to hold the entire amount. A preservative may be added to prevent bacterial decomposition but preserved specimens are not suitable for cultures or animal inoculation.

A 24-hour specimen is recommended when examination for tubercle bacilli is to be made. For tubercle and other general examinations, sterile containers are not necessary but they should be clean. For typing pneumococci and similar tests and for culturing bacteria and other microorganisms, sputum should be collected in sterile containers.

When single specimens are collected for bacteriological examination, proper collection is imperative. The patient should first rinse his mouth thoroughly with water. He should then cough deeply and expectorate into a sterile container. A wide-mouthed bottle, a Petri dish or a cardboard container will be suitable for this purpose. Waxed cardboard containers may be burned in the disposal of the specimen. If bottles are used, they should be fitted with new corks. The best time to obtain the specimen is in the early morning because the output of sputum is greater, representing the pulmonary secretions accumulated during the night. Furthermore, such specimens usually contain more bacteria.

Sputum should be examined as soon as possible after collection since changes occur in time that may render the specimen unfit for examination. Sputum more than 24 hours old is not suitable for examination.

PHYSICAL CHARACTERISTICS

The physical properties of sputum are determined mostly by inspection of the specimen or a representative portion.

Volume

This is determined by measuring the total sputum output over a twenty-four hour period. Large volumes of sputum (over 100 ml.) occur in pulmonary edema, bronchiectasis, advanced pulmonary tuberculosis, pulmonary hemorrhage, lung abscess, pulmonary gangrene and bronchial asthma. A large volume may be caused by accumulation from an extrapulmonary collection of pus or fluid which connects with a bronchus, as in sub-phrenic abscess or empyema. The volume is an index in prognosis. Increasing volumes usually indicate progression in the disease. A gradual decrease in volume may indicate healing but a sudden decrease usually indicates obstruction of drainage and generally is followed by constitutional symptoms, if drainage is not reinstituted.

Consistency and Appearance

The consistency of sputum varies from that of fluid to a thick mucoid mass with elastic properties which may be almost rubber-like in quality. Pneumonia frequently gives rise to a tenacious sputum. Descriptive terms are liquid, mucoid, tenacious, or glairy.

Sputum may appear clear and watery or may be quite opaque from cellular material. Often the bulk of the sputum is fluid with opaque masses or particles suspended in it. These opaque particles may be masses of pus or epithelium, bronchial casts, Curschmann's spirals, Dittrich's plugs, broncholiths, caseous matter or food substances. Particular attention should be paid to the solid appearing material in sputum. The presence of air results in a frothy appearance. The appearance may be described in appropriate terms such as mucoid, purulent, muco-purulent, bloody, serous, frothy, etc.

Color

The color of sputum is determined by the contained material. A great variety of colors are seen. Mucoid sputum is practically colorless but pus imparts a yellowish color. Orange to rusty colored sputum is due to decomposed hemoglobin. Hematin imparts a brown color and fresh blood gives it a red color. The amount of blood may vary from a few red streaks to a large bloody mass. The presence of iron oxides imparts to sputum a reddish tint. Sputum mixed with the blood of

hematemesis is dark or black in color and other black colors may be caused by carbon or coal dust. Lesser amounts of these substances give a gray appearance. Green sputum is the result of bile pigments, old pus, or chromogenic bacteria such as *B. pyocyaneus*.

Odor

Fresh and undecomposed sputum usually has no odor but if bacterial decomposition has taken place, either within the body or after expectoration, there will result varying types of odors, such as sweet, putrid, sour, or cheese-like. Suppurative conditions of the lung such as abscess, bronchiectasis, gangrene and tuberculosis with extensive cavitation are productive of the most foul-smelling sputa.

Stratification (Formation of Layers)

Most diseases producing large amounts of sputum will show layers or separate into strata of the sputum on standing in a tall cylinder. Usually three layers can be discerned: a frothy layer on top, a more or less clear layer in the middle, and a bottom layer containing the dense mucus and cellular elements. Such sputum may be obtained in abscess or gangrene of the lungs and in bronchiectasis.

Specific Gravity

This fluctuates considerably, not only in different cases of the same disease, but sputum from the same patient may show considerable fluctuation in different stages of the disease. It is not, therefore, of great diagnostic importance. Clifford states that Cossel, who found the specific gravity of sputum by heating it to a temperature of 60°C., at which it became liquified, compiled the following table of the maximum, minimum and average specific gravity to be found in different diseases:

	Maximum	Min mum	Average
Bronchitis	1014.0	1004.3	1008.3
Emphysema	1013.0	1006.2	1010.6
Lung abscess	1018.0	1015.5	1016.7
Pulmonary tuberculosis	1026.0	1008.0	1012.0
Pneumonia	1020.4	1010.4	1013.9
Fibrinous bronchitis (Lepine)			1008.0

Reaction

When fresh, it is generally alkaline. It may be acid where cavitation occurs and in conditions where sputum remains in the lung for a long period.

MACROSCOPIC EXAMINATION

Significant structures may be identified by the unaided eye or supplemented by means of a hand lens; however, they are seen to a better advantage with the aid of a microscope.

Macroscopic examination may reveal the following structures.

Curschmann's Spirals

These are yellowish-white masses composed of a central thread around which is wound either loosely or tightly a mass of delicate fibrils frequently embedded in mucus. When observed microscopically, leukocytes and Charcot-Leyden crystals often are seen adhering to the spiral. Curschmann's spirals are suggestive of bronchial asthma but also may be found in certain peneumonias, acute bronchitis and in chronic pulmonary tuberculosis.

Dittrich's Plugs

These are yellowish or gray caseous bodies, formed in the bronchi which sometimes may be expectorated alone. They vary from pinhead to bean size and are characterized by a very putrid odor, especially noticeable when crushed. Microscopically, they consist of granular debris, fat globules, fatty acid crystals, large clumps of bacteria and occasionally contain leptothrix organisms.

They are most common in chronic bronchitis, bronchiectasis and bronchial asthma but may be expectorated by apparently healthy individuals. The laity commonly associate them with pulmonary tuberculosis in which actually they are seldom found. Somewhat similar caseous masses are formed in crypts of the tonsils and may be mistaken for Dittrich's plugs, and, in fact, are sometimes included under this classification.

Bronchial Casts

These casts of the bronchi are white or grayish, but may be reddish-brown when mixed with blood pigment. Frequently they are composed of fibrin and vary in size and appearance from small threads to large tree-like branching masses depending upon the size of the bronchi in which they were formed.

They may be fibrinous, hemorrhagic or mucus in character and frequently are rolled into balls or tangled masses which are recognized best by floating out in water over a black background. They occur

most frequently in cases of fibrinous bronchitis, pneumonia during the stage of consolidation, chronic cardiac diseases, tuberculosis, chronic lung conditions secondary to mediastinal compression and in cases of diphtheria when there is involvement of the trachea and bronchi.

Their expulsion is similar to that of a foreign body and careful search should be made for them in the sputum when there is hemoptysis, intense dyspnea and suffocation out of proportion to the clinical findings. In pulmonary tuberculosis, casts may be expelled during the course of or at the end of a hemorrhage. They may contain tubercle bacilli for which the usual microscopic examination should be made (see fig. 22.2, item 11).

Pneumoliths (Lung Stones)

These are small concretions of calcarious material varying in size from fine sand-like particles to large stones and are designated as pneumoliths, lung stones, lung calculi or broncholiths. Formation is probably due to necrosis of infected tissue and deposition of calcium salts in the stagnant contents of a bronchus or cavity. They are not infrequently coughed up during the course of chronic tuberculosis. They may result from small foreign bodies, bits of clothing, etc., being carried into the lung tissues or bronchi by various injuries. After a long period of time, they may become encrusted with calcium salts, and upon ulceration become free and are expectored, usually with hemorrhage. Symptoms are absent if these concretions remain quiescent. When they produce active inflammation, clinical symptoms are characterized by paroxysmal cough, wheezing and dyspnea. Bronchitis or asthma and hemoptysis may accompany their expulsion and is referred to as "stone asthma," bronchopulmonary lithiasis or as colique bronchique (Fig. 22.1).

Cheesy Masses

These are small particles of caseous material varying from the size of a pinhead to that of a bean, consisting of fragments of necrotic pulmonary tissue or bits of cartilaginous rings. In the presence of considerable pus, they are yellow and not easily seen. They may be dark due to decomposed blood or other pigments. Most commonly they are found in the sputum in pulmonary tuberculosis, abscess and gangrene. Macroscopic identification followed by microscopic examination are important as they are most apt to contain tubercle bacilli and elastic fibers. In the absence of gangrene, they may be large enough to be recognized by the

naked eye. In tuberculosis, they may be so small that the use of a hand lens is necessary.

Foreign Bodies

These consist of particles of clothing, etc., carried into the lungs by gunshot or other penetrating wounds, or objects such as peanuts, buttons, marbles, etc., which children may put into their mouths and then inhale. They may remain in the lung for years, finally ulcerate into a bronchus and be expectorated.

MICROSCOPIC EXAMINATION

This very significant part of the examination of sputum, although simple, is sometimes neglected or poorly performed. Much additional valuable information may be gained by the careful selection of choice particles and examining them in (A) unstained preparations and (B) stained preparations.

Unstained Preparations

Select cheesy masses, purulent or any solid particles, "rusty" portions and any suspicious structures. These are teased or cut out with the aid of two toothpicks and placed on a clean slide. A coverglass is then applied and the unstained specimen examined with 16 mm. and 4 mm. objectives. Light should be decreased by a partial closure of the diaphragm. The oil immersion lens should not be used for this purpose.

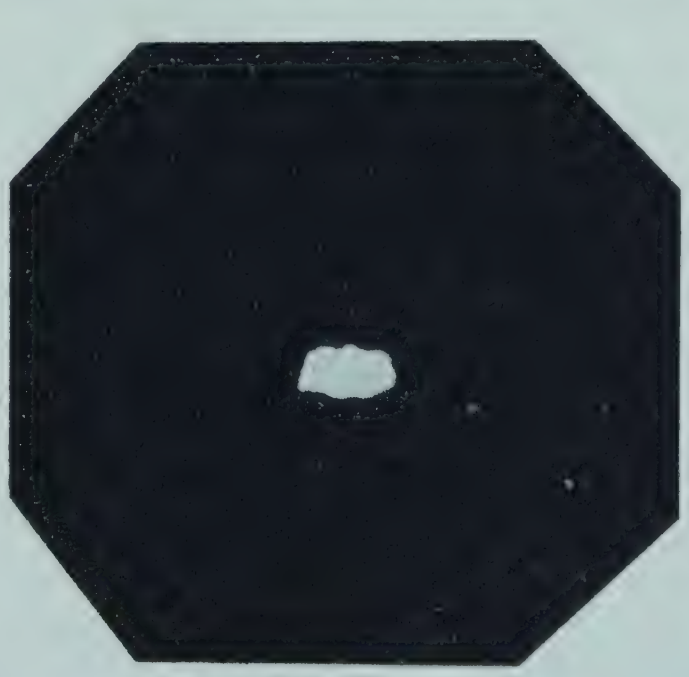


FIG. 22.1. Concretion resembling small piece of coral found in the sputum from a case of lung abscess. (Clifford Randall: The Sputum, The Macmillan Company.)

The inoculation needle used for bacteriological work is not satisfactory for transferring material because it is not stiff enough. A little practice with toothpicks will result in satisfactory transfer. *Never dip the slide or coverslip into the sputum and never allow any of the sputum to run over the edges in spreading.*

The most important structures to be seen in unstained specimens are Curschmann's spirals, elastic fibers, certain crystals such as Charcot-Leyden, fatty acid, cholesterol, leucine, tyrosine and inorganic salts. In addition to these, the so-called "heart-failure" cells, dust cells, myelin globules, fungi and animal parasites (ova, larvae and trophozoites), may be seen.

1. Curschmann's Spirals. These and the microscopic structures accompanying them with the consideration of their clinical significance have been described under Macroscopic Examination.

2. Elastic Fibers. They appear as slender, curled, highly refractive, branching fibers of uniform diameter (Fig. 22.2, item 10).

They may be seen as a network or in bundles, and sometimes retain their alveolar arrangement. They are differentiated from vegetable fibers, such as cotton or molds or hairs, by the absence of alveolar formation. The fibers may be separated for study by dissolving the sputum in equal parts of sodium hydroxide, boiling, centrifuging and examining the sediment.

They are derived from the alveoli, bronchi or blood vessels, and their presence in sputum indicates destruction of pulmonary tissue. They may be found in advanced tuberculosis, gangrene of the lung, lung abscess, ulcerating bronchiectasis or ulcerating malignancy.

3. Crystals. a. *Charcot-Leyden Crystals.* These appear as colorless pointed hexagons, or they may appear quite needle-like, are soluble in water and acetic acid and may be stained with eosin.

They are often absent in fresh sputum but make their appearance after the specimen has stood for some time. They are derived from the disintegration of eosinophiles, are associated with bronchial asthma and may be seen embedded in Curschmann's spirals (Fig. 22.2, item c).

b. *Fatty Acid Crystals.* They resemble long colorless needles, straight or curved, and are found singly, in tufts or sheaves (Fig. 22.2, item a). They are insoluble in water and acids but soluble in ether, hot alcohol, chloroform and alkalies which make them easily distinguishable from the elastic fibers. They are usually associated with chronic pulmonary

tuberculosis, gangrene of the lung, putrid bronchitis and bronchiectasis. The presence of single needles is comparatively of little importance.

c. *Cholesterol Crystals.* These are colorless and transparent, appearing as large or small, thin rhombic, rectangular or oblique plates with notched edges (Fig. 22.2, item d).

They are generally found in chronic lung abscess, empyema, chronic tuberculosis, and in cases of liver abscess opening into the bronchi.

d. *Leucine and Tyrosine Crystals.* They are found less frequently than the other crystals mentioned. These crystals represent decomposition of protein.

Leucine crystals are gray or yellowish spheres, somewhat resembling fat cells, and are found either singly or in clumps. They may sometimes



FIG. 22.2. Objects found in the sputum (Landois). 1, Detritus and dust particles; 2, pigmented alveolar epithelium; 3, fatty degenerated and partially pigmented alveolar epithelium; 4, alveolar epithelium showing myelin-degeneration; 5, free myelin forms; 6, 7, desquamated ciliated epithelium, partly changed and deprived of its cilia; 8, squamous epithelium from the mouth; 9, leucocytes; 10, elastic fibers; 11, fibrinous cast of a small bronchus; 12, leptothrix buccalis, together with cocci, bacilli and spirochete; a, fatty acid crystals and free fatty granules; b, hematoidin; c, Charcot's crystals; d, cholesterin. (From Webster: Diagnostic Methods, Chemical, Bacteriological and Microscopical, The Blakiston Company.)

appear as discs having a concentric arrangement resembling the transverse cut of a tree trunk, the outer ones having edges like fine lace.

Tyrosine crystals are fine silky needles, which may appear singly, in groups or arranged in single or double tufts, point to point, often having the shape of a rose window or sheaves.

For illustrations of both types of crystals, see Chapter 21, Renal Function Tests and Urine Examinations, Plate 21.I.

These crystals are more readily detected if the sputum is evaporated in the air so that they separate. Leucine crystals are seldom found unless this is done. Both types of crystals may be found after the rupture of an empyema into the lung, and occasionally after the perforation of a liver abscess into the lung.

e. *Inorganic Salt Crystals.* These are of little or no clinical importance.

4. Pigmented Cells. a. *"Heart-failure" Cells.* These cells contain hemosiderin and have been designated as "heart-failure" cells because they are most frequently found in long continued passive congestion of the lungs resulting from poorly compensated heart disease.

To identify more clearly, add one drop of 10 per cent potassium ferrocyanide, and after a few minutes add one drop of 0.1 NHCl. Iron containing pigment shows the characteristic Prussian blue color of ferriferrocyanide.

These cells are found in chronic passive pulmonary congestion due to cardiac decompensation, pulmonary infarction and for a time following pulmonary hemorrhage (Fig. 22.2).

b. *Dust Cells.* These are similar to, but less important than, the "heart-failure" cells. They contain black or brownish-black angular granules, and are seen in the sputum in anthracosis. Cells similar to these containing smaller carbon particles are often seen in the morning sputum of individuals living in regions with smoke-laden atmosphere, and in that of individuals who inhale tobacco smoke to excess.

5. Myelin Globules. These usually appear as uncolored objects, irregularly shaped, oval, round or pear shaped in groups of various sizes, which are highly refractile, having a greenish sheen. They frequently show spiral markings or concentric rings and often resemble fat droplets. Care must be taken not to confuse them with blastomyces.

They may be seen in abundance in the scanty morning sputum of apparently healthy individuals, but may be especially abundant in the

mucoid sputum of bronchitis. They have little or no clinical significance (Fig. 22.2).

6. Fungi. Occasionally, fungi may be found in the sputum of persons whose clinical picture resembles that of pulmonary tuberculosis.

These organisms are usually sought in unstained moist preparations, cleared with 10 per cent potassium hydroxide which dissolves the cellular debris, making the fungi more visible. A coverglass is applied to such preparation and gently heated over a slow flame. Examination of the preparation is usually made under the 16 mm. and 4 mm. lens. Also, Ajello has shown that fungi frequently survive the concentration techniques employed to demonstrate *M. tuberculosis* in sputum and furthermore that these fungi grow fairly well on the various culture media employed for *M. tuberculosis.*

It is not always an easy matter to establish the etiological relationship of these organisms when found in sputum. Isolation and study on special culture media, animal inoculation and x-ray may be necessary. For further information, see Chapter 13, Medical Mycology.

7. Animal Parasites. Of the animal parasites which may be encountered in the sputum, the following should be noted: Larvae of *Nacator americanus*, Strongyloides and Ascaris, ova of the lung fluke Paragonimus, *Endamoeba histolytica* from lung abscess or perforating abscess of the liver, flagellate protozoa such as Trichomonas. Fragments of the wall of an echinococcus cyst or the contents, especially the hooklets, may be seen in the sputum, being derived from a perforating cyst of the liver, kidney or lung. A description of these parasites is given in Chapter 14, Medical Parasitology.

Stained Preparations

Cells and bacteria are the principal structures in sputum best identified on stained smears. The various structures described under unstained specimens may be stained and thereby identified more readily. In addition leukocytes, erythrocytes, epithelial cells and bacteria are identified by appropriate staining. Neutrophiles, lymphocytes, eosinophiles, endothelial leukocytes and erythrocytes are identified easiest using Wright's or Giemsa stain. Bacteria are identified by employing the usual bacterial stains.

1. Leukocytes. Polymorphonuclear neutrophiles often are partially disintegrated and so difficult to identify. Their presence indicates a pyogenic infection.

2. Eosinophiles. These may be numerous in bronchial asthma and while highly characteristic are not pathognomonic of that condition.

3. Lymphocytes. These predominate in early or mild cases of uncomplicated tuberculous infection, but a few are present with ordinary purulent infections.

4. Endothelial Leukocytes. See pigmented or "heart-failure" cells.

5. Erythrocytes. Blood cells appear as circular, flattened or biconcave disks, yellowish-green in color. Care must be taken not to confuse these cells with yeast-like bodies or fungus spores which sometimes grow in old corks of the specimen bottles. When erythrocytes are present in large numbers, evidence of their presence may be recognized macroscopically.

Erythrocytes may be present in almost any sputum, often representing a contamination. When consistently present in large numbers, they represent hemorrhage or exudation. Examples are the rusty sputum of pneumonia, hemorrhage from a tuberculous cavity, and chronic ulcerative conditions of the lung. They may be recognized without staining in fresh sputum or by the usual staining methods employed for examining blood smears.

6. Epithelium. Recognition of these cells is important in determining the source of origin or sputum.

Squamous cells are large, flat, polygonal cells with a fairly small nucleus (Fig. 22.2). They are derived from the mouth, pharynx, and larynx, and are quite numerous in laryngitis and pharyngitis.

Cylindric cells are usually not abundant and not easily identified because they are much altered from their original form, being usually round and swollen. They are derived from the nose, trachea and bronchi. Cylindric cells are sometimes seen in bronchial asthma and acute bronchitis.

Alveolar cells are somewhat large, round or oval, containing one or two rounded nuclei, and are approximately 3 to 6 times the diameter of a red blood cell (Fig. 22.2). Their origin is presumably the pulmonary alveoli, and are likewise present in normal sputum, but are numerous in bronchitis, in general inflammatory conditions of the lung and in irritation of the respiratory tract.

BACTERIOLOGIC EXAMINATION

Various types of bacteria are found in the sputum. A number of species coming from the mouth are saprophytes representing contamination.

Staphylococci, streptococci, pneumococci, leptothrix, borrelia of Vincent and various treponema (macrodentium, microdentium, mucosum) may come from normal, but ill kept, mouths.

Presence of tubercle bacilli (mycobacterium tuberculosis) is evidence of tuberculosis somewhere along the respiratory tract, most probably in the lung. The tubercle bacillus may also cause pneumonia, which may be lobar or bronchial.

Pneumococcal pneumonia is the commonest type of pneumonia and comprises the greater bulk of acute infections of the lung.

Pneumococci are responsible for lobar pneumonia in about 96 per cent of the cases, while hemolytic streptococci, Friedländer's bacillus (*Klebsiella pneumoniae*), *Hemophilus influenza*, staphylococci and mixed infections are responsible for about 4 per cent. Pneumococci are likewise responsible for about 50 per cent of the broncho pneumonias, streptococci 30 per cent and 20 per cent are due to miscellaneous organisms. According to Kolmer, about 25 per cent of the atypical pneumonias are believed to be caused by Beta hemolytic streptococci of the Lancefield Group A and are usually secondary to measles, pertussis, influenzae, streptococcus throat infections or scarlet fever. About 10 per cent may be caused by *Staphylococcus aureus* and are usually secondary to similar infections. Both streptococci and staphylococci may be superimposed upon pneumococcal or other pneumonias. Other organisms that may be involved are *H. influenzae*, *H. pertussis*, *Past. pestis* (pneumonic plague), *Past. tularense*, *N. catarrhalis*, *N. intracellularis*, *Gaffkya tetragena*, *C. diphtheriae*, *B. anthracis* (wool sorters disease), *E. typhosa*, *Esch. coli*, *S. dysenteriae*, Brucella (especially melitensis), *A. mallei*, *Ps. aeruginosa* and *Pr. vulgaris*.

Many of these species may cause abscess of the lung as a sequence to lobar or broncho pneumonia. This is particularly true of pneumonias due to hemolytic streptococci and staphylococci. Kolmer states that undoubtedly the most frequent micro-organisms responsible for lung abscesses are aerobic and anaerobic hemolytic streptococci, *S. viridans*, anhemolytic streptococci, hemolytic *Staphylococcus aureus* and *albus*, pneumococci, *H. influenzae*, *Kleb. pneumoniae*, Bacteroides and *Bacterium melaninogenicum*, along with *M. tetragenus* (Gaffyka tetragena), *N. catarrhalis* and other diplococci of minor importance, and frequently diphtheroid bacilli, *Borrelia vincentii* or other spirochetes in association with various types of *B. fusiformis*.

Any organism, alone or in combination, causing lung abscesses may

likewise be responsible for gangrene of the lung and bronchiectasis, especially anaerobic bacteria, *Borrelia vincentii*, other spirochetes and fusiform bacilli.

Because of their clinical importance and the frequency with which the tubercle bacilli and pneumococci are met with in the examination of sputum, they deserve special consideration in this chapter. For description, methods of staining, isolation and identification, see Chapter 12, Medical Bacteriology. For colored plates showing the staining of the tubercle bacilli, pneumococci, staphylococci, streptococci, *Borrelia vincentii* and *Klebsiella pneumoniae*, see Chapter 17, Examination of Transudates, Skin and Mucous Membranes.

Detection of M. tuberculosis (Tubercle Bacilli)

Preparation and Staining of Smears

Pour the sputum into a Petri dish and place it on a black background. Pick out the opaque particles, particularly bits of caseous material, and transfer to clean slides. For this purpose a pair of tooth picks is very convenient. Several such particles should be examined. Smears are then made by rubbing the material back and forth on the slide, using a tooth pick or wire loop. Smears about an inch and a half long and about one half inch wide are best. Stain by the Ziehl-Neelsen method.

The property of retaining the carbolfuchsin stain in the presence of acid is spoken of as an "acid-fast" characteristic. It is dependent on the presence in the cell of a waxy substance called mykol. This property is not confined to tubercle bacilli but is shared by the leprosy bacillus, smegma bacillus and a number of other organisms.

When tubercle bacilli are present, they appear as slender, straight or curved rods, staining a solid red color or perhaps showing a beaded appearance. They may appear in groups (see plates in Chapter 7).

Fluoroscopic Method

Richards and Miller have developed a simple and efficient method in the examination for acid-fast bacilli, employing the principle of fluorescence microscopy. The bacteria are stained with a fluorescent dye, Auramine O, which when irradiated with ultraviolet light re-radiates as yellow light. When viewed with a fluorescence microscope, the bacteria appear as brilliant yellow organisms against a black or nearly black background.

Advantages. The method is simple, requires no counter staining and

consumes about one-third less time for preparing and reading smears. When bacteria are few and far between, more positive smears will be found than with the usual Ziehl-Neelsen method of staining.

Interpretation

Acid-fast rods in the sputum may be considered to be tubercle bacilli. In the presence of clinical tuberculosis their absence from sputum may mean that either the concentration of organisms is too low for detection by smear methods or the lesion is not breaking down. It may indicate that the process is in the proliferative rather than the exudative phase. Repeated tests are often necessary to reveal the organisms. It has been estimated that at least 100,000 bacilli per cubic centimeter of sputum must be present for their detection in the average smear. If a quantitative estimate is required, the average number per field should be reported. To insist that a positive report must be based on the finding of a certain minimum number of bacilli is illogical since only one or more organisms found is adequate for a diagnosis.

Gaffky's Method for Recording Number of Acid-Fast Bacilli (Brown Modification)

The following method of recording the number of acid-fast bacilli observed on each slide is widely used.

Gaffky I—only 1 to 4 bacilli in the whole preparation.
Gaffky II—only 1 bacillus on average in many fields.
Gaffky III—only 1 bacillus on average in each field.
Gaffky IV—2 to 3 bacilli on average in each field.
Gaffky V—4 to 6 bacilli on average in each field.
Gaffky VI—7 to 12 bacilli on average in each field.
Gaffky VII—13 to 25 bacilli on average in each field.
Gaffky VIII—about 50 bacilli on average in each field.
Gaffky IX—about 100 bacilli on average in each field.
Gaffky X—enormous masses of bacilli on average in each field.

Concentration Method (Petroff)

A quantity of sputum is poured into a sterile tube and an equal amount of normal sodium hydroxide solution is added. The tube is then incubated at 37.5°C. for half an hour with occasional shaking. An equal amount of N/1 HCl is then added to neutralize the alkali. Centrifuge the solution at high speed to throw down the bacteria. The sediment can then be used for smears, animal inoculation and cultures. It is

advisable to smear the sediment in a small drop of 1:10 egg albumin which causes the material to adhere to the slide.

The advantage of this method over some of the other concentration methods is that it can be carried out quickly and does not require washing of the sediment which sometimes results in loss of organisms. The sodium hydroxide destroys the viscosity of the sputum and also kills other bacteria thereby producing a suitable digest for animal inoculation or culture.

Guinea Pig Inoculation

Suitable material can be prepared by concentration as described in the preceding section. It is not advisable to use untreated sputum for inoculation because of the danger of pyogenic infections and loss of the animal which is a common result when fresh, unaltered sputum is used.

Other Bacteria

Pneumococci, streptococci, staphylococci, etc., can be recognized on smears stained with Gram's stain, carbolfuchsin or methylene blue, while *C. diphtheria* is better stained by Kenyon's stain.

SPECIAL EXAMINATIONS

See Chapter 12, Medical Bacteriology, for descriptions of cultural characteristics, typing of pneumococci, etc. Also, see Chapter 13 for characteristics of medically important fungi found in sputum.

CHEMICAL EXAMINATION

Albumin

To 5 ml. of fresh sputum in a test tube, add 10 ml. of 3 per cent acetic acid. Shake well and filter. Mucin is removed by this method. Determine albumin in the filtrate by the use of the quantitative methods as employed for urine.

In tuberculosis and pneumonia, albumin varies from 0.1 to 0.3 per cent or 1 to 3 grams per liter. Only traces are found in chronic bronchitis and asthma.

Fat

Fat can be readily demonstrated in all sputum specimens but has no clinical significance.

Blood

This is determined by the benzidin test or other methods used for detection of occult blood for the technique (see Chapter 25, Feces).

CHARACTER OF THE SPUTUM IN VARIOUS DISEASES

Bronchial Asthma

The sputum is scanty, grayish-white, semi-transparent, viscid, and tenacious, containing eosinophils, which are diagnostically important. Curschmann's spirals and Charcot-Leyden crystals are sometimes seen.

Acute Bronchitis

Early in the disease the sputum is scanty, tenacious, mucoid and white, containing a few leukocytes and epithelial cells, and frequently blood streaked. Later it becomes more voluminous, muco-purulent, yellowish or gray, containing epithelial cells, pus cells and bacteria of various types.

Fibrinous Bronchitis

The sputum is mucoid and abundant early in the disease, containing leukocytes, epithelial cells, and fibrinous coagulum often seen as bronchial casts rolled in a mass difficult to detect. It may also contain blood and mucus.

Bronchiectasis

An abundant purulent sputum is formed, the amount expectorated varying with posture. Very often it has a putrid, offensive odor, is grayish-green in color and usually separates in distinct layers on standing. Blood may be present when there is marked tissue destruction or hemorrhage.

Pulmonary Abscess

This is a purulent, yellow or green sputum, varying in odor from sweetish to most offensive. The sputum is scanty until the abscess ruptures into a bronchus. When rupture occurs, a large amount of creamy foul-smelling pus, often containing lung tissue fragments, is expectorated. Pus cells are numerous and elastic fibers and erythrocytes may be seen.

Pulmonary Gangrene

This may be produced by conditions similar to those giving rise to pulmonary abscess and may follow a pulmonary abscess. Sputum is large in volume, fluid, green to brown in color and is characterized by a marked fetid odor. It undergoes stratification on standing, and elastic fibers, necrotic tissue, and erythrocytes may be seen. Pus and epithelial cells are not numerous. Bacteria of various types are usually present, and careful examination may reveal many characteristic fusospirochetal forms of Vincent.

Lobar Pneumonia

The sputum usually becomes rusty and may be very tenacious, with orange to green colors sometimes seen. Pus cells, epithelium and pneumococci predominate in the stained smears. Fibrinous casts are common.

Bronchopneumonia

A muco-purulent, viscid, not over-abundant sputum, containing pus cells, erythrocytes and bacteria is found. Sputum in this disease is not very characteristic.

Pulmonary Tuberculosis

In early tuberculosis, the sputum is often scanty, mucoid, and may contain opaque yellow particles. In the advanced stages associated with progressive cavitation, the sputum becomes abundant and frequently contains caseous particles. A thorough microscopic examination of these opaque particles often reveals tubercle bacilli. These may appear early or late in the disease or may occur intermittently. Blood may or may not be present.

Pulmonary Edema

Due to the large volume of serous exudate, the sputum is abundant, watery and frothy. There may be 1 to 2 liters per day in severe cases. It varies in color from pink to dark brown and contains hyaline masses, varying numbers of red cells, a few leukocytes and epithelial cells. Many pneumococci may be present if the edema follows pneumonia.

Chronic Passive Congestion

When due to mitral heart disease, especially mitral stenosis, the presence of blood may be one of the distinguishing features. It may be

streaked, giving a rusty appearance to the sputum, or it may occur in dark masses mixed with mucus. Frank hemorrhages may occur. "Heart-failure" cells and erythrocytes are found.

Infarction

When uncomplicated, the sputum is small in amount, tenacious, mucoid and bloody. It may contain isolated masses resembling blood which, on examination, prove to be tenacious mucus mixed with blood. These masses are bright red early in the disease and become dark red as the disease progresses. The sputum may become purulent if secondary infection occurs. Large numbers of pigmented alveolar cells and erythrocytes are present. Leukocytes are few in number unless some chronic lung infection has occurred prior to infarction. A striking feature in early infarction is the absence of large numbers of bacteria or of any one specific organism and may be a point of differentiating infarction and pneumonia.

After the initial infarction when the blood clot in the lungs breaks down offering an excellent culture medium which, particularly if the embolus is septic, results in the presence of a large number of bacteria in the sputum. The sputum then becomes more abundant and purulent and may have a foul odor with mixed bacteria, leukocytes and hematoidin crystals. A bloody purulent sputum containing hematoidin crystals and large numbers of streptococci is suggestive of abscess formation and possible secondary extension to the pleura, with resulting empyema.

Lipid Pneumonia

Macrophages containing liquid petrolatum or vegetable and animal oils may be demonstrated. Sudan IV (Scarlet Red) and osmic acid stain both animal and vegetable oils. Mineral oils are stained only with Sudan IV.

Sputum Following Therapeutic Serum Treatment

Antipneumococcic serum results in clumping of specific pneumococci and increased phagocytosis.

Sputum Following Sulfonamide or Antibiotic Therapy

In penumonia the number of organisms in the sputum decreases within 24 hours following specific antibiotic or sulfa drug therapy. Pneumococci show chaining and may vary in size, shape and staining reaction.

INTERPRETATION

Careful examination of the sputum and considered evaluation of the findings can be most helpful in the diagnosis and treatment of pulmonary disease. The specimen is generally easily obtained, the examinations simple for the most part, and the information gained frequently most helpful. A negative result from a single examination is of little significance in ruling out any particular diagnosis. Frequently repeated examinations are necessary, particularly in searching for *M. tuberculosis*. Sputum is generally contaminated by the saprophytic organisms from the throat and mouth and this must always be remembered in evaluating the results of a sputum examination.

Many cases, largely children, have no sputum and cannot or will not cooperate in obtaining a satisfactory specimen. Here throat swabs and the flecks of material obtained from the accompanying coughing are readily obtained for examination. Such specimens are particularly important in acute pulmonary infections. Numerous individuals, particularly children, swallow their sputum and when tuberculosis is suspected in such individuals gastric washings should always be obtained.

Changes in the course of pulmonary diseases are frequently reflected early by changes in the amount and/or character of the sputum. Therefore, it behooves the physician to carefully follow the sputum for such changes.

Sputum and Bronchial Aspirates in Malignant Disease of the Lung

GENERAL CONSIDERATIONS

With the present high incidence and mortality of carcinoma of the lung, improved early diagnostic and therapeutic techniques have been sought for some time. Development and wide use of the surgical technique of lobectomy has increased the urgency for improved early diagnostic procedures of high specificity.

Papanicolaou, in 1946, reported that he found malignant cells in the sputum of 88 per cent in a series of 25 carcinoma of the lung cases. Since then numerous workers have reported similar results. Wandall reported extensive cytological studies of the sputum in 100 cases of lung carcinoma and definitely established the diagnosis in 86 per cent of the cases. Wihman and Bergstrom have reported that cytological examination of the sputum permitted an earlier diagnosis of lung carcinoma than other diagnostic methods.

SPUTUM IN MALIGNANT DISEASE OF THE LUNG

The sputum varies greatly in amount and character in lung malignancies. In fact there may be no sputum with extensive lung involvement or abundant sputum with a small irritative lesion. If the growth becomes necrotic the sputum will have a very foul odor. Blood-streaked sputum and even frank hemoptyses are common findings in lung malignancies and should not mislead the physician toward tuberculosis.

BRONCHIAL ASPIRATES

In the absence of sputum a satisfactory specimen may be obtained by bronchoscopy and aspiration of the bronchial secretions. Apparently tumor cells are being desquamated fairly regularly by malignant growths in the lung and are moved upward by the bronchial cilia. In the event little or no bronchial secretions are present 1 or 2 ml. of sterile physiologic saline may be introduced through the bronchoscope and the bronchial washings thus obtained make satisfactory specimens. Also smears from swabbing the laryngeal folds may show malignant cells. Woolner and McDonald report that sputum and bronchial aspirates are equally satisfactory for demonstrating malignant cells so there is no advantage to bronchial aspirates if the patient can produce sputum.

PREPARATION OF SPECIMENS

Fresh sputum specimens are imperative since tumor cells degenerate rather rapidly and early fixation is necessary to preserve these cells. The freshly produced sputum specimen, from deep coughing, is examined grossly and if sufficient gross particles and flecks of blood clots are present they may be transferred to slides and smears made directly. If, however, few or no gross particles are present or the specimen is thin and watery, it should be centrifuged and smears made from the sediment. A second centrifugation, mixing the sediment with 1 ml. of human blood serum, is highly advisable. In any event 4 to 6 smears should be made if there is sufficient material, fixed with a mixture of half 95 per cent ethyl alcohol and half ether for several minutes, then drained. Then flood the smears with Wright's stain for 1 minute, followed by buffered water for 10 to 15 minutes. Finally, wash with distilled water, drain, and air dry.

If there is substantial sediment or viscid lumps of sputum are present, the material may be attached to a small square of blotter by egg albumin, fixed in formalin, embodied in paraffin, and serial sections made.

These paraffin sections are stained by hematoxylin and eosin and examined for malignant cells as a regular tissue section.

EXAMINATION

The smears should be examined by the low power objective to find clumps or clusters of suspicious cells, then these cells carefully examined under oil immersion lens to study cell detail. According to Diggs, malignant cells are characterized generally as large cells with large acidophilic nuclei having a delicate chromatic pattern with nucleoli. The cytoplasm is bluish and non-granular. In groups the individual cells have a disorderly arrangement, varying in size, shape, staining and nuclear configuration and structure. Mitotic figures, multiple nuclei, naked nuclei and "nuclear clefts" may be distinguished. Chromatin material may be found outside the nucleus in the cytoplasm. The nuclei are crowded and displaced by secretory material. There are apt to be organoid arrangements and finally keratinization is frequently present.

INTERPRETATION

Using the time honored diagnostic techniques of chest x-ray and bronchoscopic biopsy most cases of primary lung cancer are diagnosed and resection therapy attempted so late that few cases survive for five years. In 1949, at the National Cancer Conference, in 548 cases of diagnosed bronchogenic carcinoma only about 35 per cent were resectable and only 7.5 per cent survived after 5 years. This most dramatically points to the need for a diagnostic technique that will early establish the diagnosis in resectable lung tumors.

The work of Papanicolaou and others has shown conclusively that malignant tumors exfoliate. Apparently the location of the tumor in the lung bears no relationship to the ability of the exfoliated cells to reach a main bronchus, although a mucous plug conceivably could temporarily prevent the passage of such exfoliated cells. In view of this it is important to examine several specimens before considering a possible case as non malignant. In this connection, Farber reported that his diagnostic efficiency rose from 55 to 90 per cent when he increased the number of specimens from each patient from 1 or 2 to 5. Therefore, it is important to emphasize that 1 or 2 negative examinations do not exclude a diagnosis of carcinoma. However, a series of negative examinations is of much more clinical significance.

Finally, it must be clearly recognized that a freshly deeply coughed up specimen is imperative, that it requires considerable technical skill

and experience to prepare good smears, that there is no substitute for patience and time in completely examining each *entire* slide, and lastly every suspicious cell should be carefully checked by an experienced pathologist before rendering a definite diagnosis. Using this technique with repeated examinations the early accurate diagnosis of primary carcinoma of the lung is possible in about 85 to 90 per cent of cases from a laboratory technical point of view.

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CHAPTER 23

GASTRIC AND DUODENAL CONTENTS

By Seward E. Miller

The Stomach

GENERAL CONSIDERATIONS

Although the stomach has numerous functions none is absolutely essential, as life can be maintained following a total gastrectomy. The stomach serves as a primary storage place and mechanical mixing chamber where food is broken up, mixed with gastric juice, and reduced to a semifluid state. Some preliminary digestive actions occur from the gastric lipase, pepsin, rennin, and the hydrochloric acid. Some minor absorption of a few substances, alcohol being among the more important, takes place directly from the stomach. The stomach also exerts a minor bactericidal action, produces considerable mucin and the intrinsic antianemic factor.

The stomach normally secretes hydrochloric acid, the enzymes gastric lipase, pepsin, and rennin, and mucin of two types—the soluble and the so-called "glairy." These secretions comprise the gastric juice which hydrolyzes some disaccharides, rennin, coagulating milk, and pepsin, starting the digestion of proteins to proteoses and peptones.

In the duodenum the semifluid food is mixed with the alkaline bile from the liver and the pancreatic juice from the pancreas. The pancreatic juice contains such a diverse array of enzymes that it is capable of almost complete digestion of all foods unaided by other enzymes. Trypsin and chymotrypsin, the proteolytic enzymes, further reduce proteins to the polypeptide stage and even a few amino acids. Pancreatic amylase breaks down starch to dextrins, then to maltose and even some glucose. Pancreatic lipase splits fats into its basic constituents, fatty acids and glycerine. In this process the bile salts are most helpful by their emulsifying action which greatly enhances the efficiency of lipase.

Examination of gastric and/or duodenal contents is of importance in a number of conditions of disturbed function of the stomach, duodenum, liver, pancreas, and the systemic condition of anemia. Only occasionally will such examinations verify or produce a specific diagnosis such as carcinoma, pernicious anemia, gallstones, or fibrocystic disease of the

pancreas. Usually the information gained can be significantly helpful when collated with other clinical information in enabling the physician to arrive at a sound diagnosis or assist him in treatment and prognosis.

Normal gastric juice contains, in addition to free hydrochloric acid, some acid in combination with protein (combined acid), the enzymes pepsin, rennin, and gastric lipase, potassium chloride, sodium chloride, phosphates, mucin, and the intrinsic erythropoietic factor. These substances are secreted continuously to a limited extent, but largely in response to certain physiologic stimuli which may be psychic (thought, sight or odor of food), the presence of food in the stomach, or the action of products of digestion in the intestine. In addition certain diseases may be associated with the presence of abnormal constituents in the stomach contents such as blood, pus, bacteria, tumor fragments or food particles.

Appraisal of the gastric contents involves the removal and examination of the fasting contents as well as that obtained after use of a test meal.

COLLECTION OF GASTRIC CONTENTS

Gastric content for examination is obtained by aspiration from the stomach through a special rubber tube, three types of which are available. The Rehfuss tube, consisting of a 36-inch length of ⅜ 18–20 F. rubber catheter tubing to which a gold or silver plated fenestrated tip is frequently attached, is passed through the mouth and is the one of choice where collection of duodenal content is desired. The Levin tube (Fig. 23.1) is designed for introduction through the nasal passages and is preferred by many laboratories. This mode of passage is particularly desirable where the tube must remain in place for periods of time. This tube is a rubber catheter similar in length to the Rehfuss tube but softer and slightly smaller in diameter. It has a slightly tapered tip provided with several openings through which contents may be aspirated. Both of these tubes are marked at intervals with a single, a double and a triple ring. As the tube is passed the proper mark at the teeth or the nostril indicates respectively the location of the tip at the cardia, in the stomach, or that sufficient tube has been inserted to allow the tip to pass into the duodenum.

The third or older type of tube (Boas tube) has a much larger bore and is made of moderately stiff rubber. It is useful for emptying and washing the stomach in cases of poisoning but is rarely used for collection of gastric content.

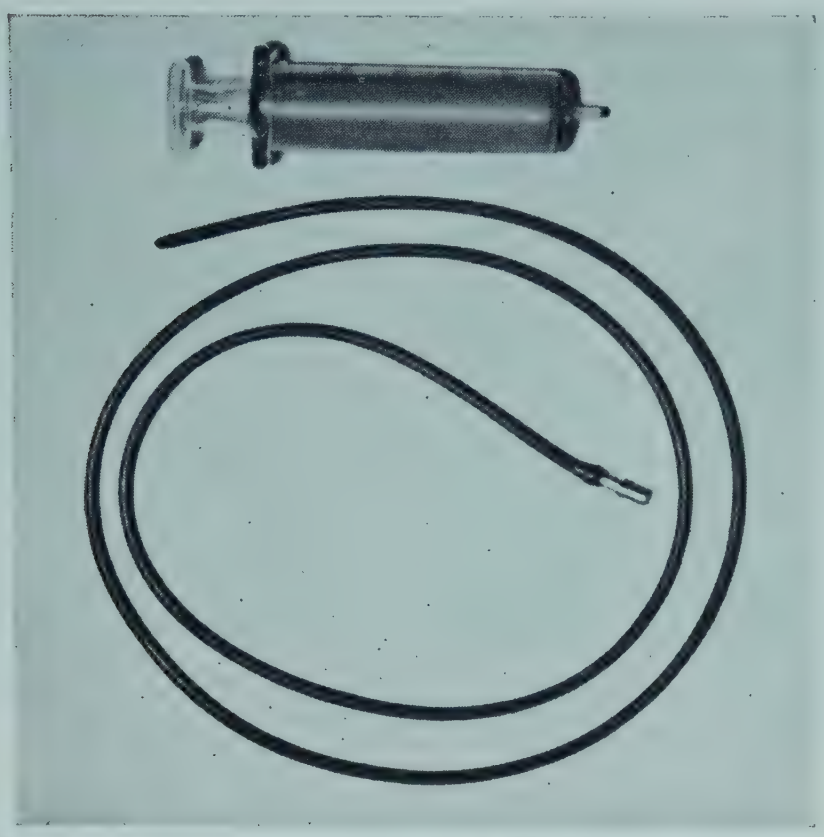


FIG. 23.1. The Levin gastro-duodenal tube which is usually introduced through the nose. A metal adapter permits the use of a glass syringe for aspiration.

Passage of the tube by mouth is facilitated by having it cold and wet. Place the tip well back in the pharynx. Instruct the patient to groove the tongue about the tube and swallow. An occasional deep breath facilitates the downward passage. When the tip is in the stomach as indicated by the appropriate ring on the tube, the gastric contents are removed by means of suction with a large syringe.

Unless there is obstruction or other defect of the nasal passages, the Levin tube is recommended for routine use. Introduction of the nasal tube is made easier by a preliminary spray of 2 per cent solution of Butyn in the nostrils and over the posterior pharynx. Lubricate the tip of tube with a few drops of glycerine.

In position shown in Fig. 23.2, introduce the tube along the floor of the nose until it is felt that the tip turns down into the pharynx. Have patient swallow repeatedly and gently push the tube down as he swallows until it is well into the esophagus, when it may be rapidly passed to the full length. The tube should be passed until the small colored ring reaches the nares. This indicates that the tip of the tube has reached the stomach. If it is desired to pass the tube further into the stomach it may be introduced for some five or six inches beyond the colored ring. A metal connection for a 25- or 50-ml. glass syringe permits aspiration of the contents or the injection of the test meal.

Patients who are particularly nervous or apprehensive should be reassured that introduction of the stomach tube is an easy procedure



FIG. 23.2. Introducing the Levin tube through the nasal passage. Left, beginning passage of tube. Right, withdrawing stomach contents.

and that no harm will result from it. At worst, it is attended only by a tickling sensation in the nose which may produce some lacrimation.

Because of the nausea that may develop during the passage of the tube, it is well to have the clothing of the patient protected by a rubber apron which can then be covered by a white towel.

At all times the passage of the stomach tube must be carried out with considerable caution, especially to avoid trauma that may cause bleeding in the esophagus or stomach. During the passage of the tube the patient should be instructed to breathe deeply as this helps to overcome nausea. After the tube has been successfully passed into the stomach it can remain there for as long as several hours with very little discomfort to the patient.

With these small tubes, it is well to remember that the tip of the tube may coil upward after entering the stomach. The tube should be raised and lowered for an appreciable distance a number of times while continuing the aspiration in order to empty the contents completely. The rapid injection of air may clear the tube of obstructing food or thick mucus if the tube appears to be blocked.

COUNTERINDICATIONS

Although the passage of a gastric tube is not hazardous, there are certain conditions which contraindicate the procedure as a routine diagnostic measure. These are stenosis or malignancy of the esophagus, aortic aneurysm, esophageal varices or diverticuli, recent severe gastric hemorrhage and cardiac decompensation. In pregnant or severely ill persons a tube should be passed only upon urgent indication.

TEST MEALS

The fasting gastric juice normally is not representative of the true secretory capacity of the stomach. Therefore a stimulus in the form of a test meal is given. Many types of test meals have been devised which utilize the stimulus of food, fluid, histamine, or insulin. Neutral red excretion may also be determined in conjunction with stimulation and is an indicator of secretory ability.

The three forms of gastric stimulation in common use are the Ewald meal, the alcohol meal and histamine injection.

The original *Ewald meal* consisted of a slice of dry toast and a cup of tea without sugar or cream. In its present modified form, a slice of soft bread (with the crust removed), or four soda crackers, and two glasses of water are given on an empty stomach. After one hour the total gastric contents are removed. This meal produces cloudy gastric fluid and in many instances fails to demonstrate the true secretory capabilities of the stomach. Absence of free hydrochloric acid with this type of stimulus cannot be considered a true achlorhydria.

The *alcohol meal* which is preferable to food meals consists of 50 ml. of 7 per cent ethyl alcohol. This is injected through the tube after the fasting contents have been removed. "Fractional" removal of the contents is usually employed, 10 ml. portions being aspirated every 15 minutes for one hour. The samples are analyzed separately and a secretory "curve" plotted. This method of stimulation provides clear material for analysis which does not require filtration. The use of the alcohol meal elicits a much greater secretory response than the Ewald meal and is recommended for routine stimulation when histamine is not employed.

Histamine phosphate, injected subcutaneously in doses of 0.19 mg. (equivalent to 0.1 mg. histamine) per 10 kg. of body weight produces maximum stimulation of the gastric mucosa and is the most satisfactory stimulant of gastric secretion. (Histamine phosphate may be procured in ampules containing 1 ml. of a 1:1000 solution.) As the drug may produce abdominal pain, flushing of the face and an accelerated pulse, it is better to use a smaller dose on the first trial (0.25 ml. of 1:1000 solution).

The injection is made after the tube has been passed and the fasting contents removed. After injection, the tube is left in place and 5-ml. samples aspirated every 15 minutes for one hour. Under unusual conditions aspiration may be continued at intervals for 3 hours or until the stomach is empty. Samples are analyzed separately and acid content plotted as a curve.

Histamine stimulates the salivary glands as well as the stomach and all salivary secretion should be expectorated. If swallowed the alkaline saliva will neutralize an indeterminate amount of acid with resulting inaccuracy.

Histamine stimulation is essential in suspected cases of pernicious anemia where the demonstration of true achlorhydria is so significant a finding.

Insulin stimulates the flow of gastric juice through the effect of the hypoglycemia upon the vagal venters. Rush regards the secretory response following its use as greater than that with alcohol and as satisfactory as with histamine. No ill effects were noted with its use in a series of 200 patients.

After the fasting gastric contents are aspirated, 1 unit of regular insulin per 10 kg. of body weight is injected intravenously and 10-cc. samples of gastric fluid are removed every 15 minutes for 1 hour or longer. The samples are analyzed separately and the acid content plotted as a curve.

Neutral red excretion is determined by injecting intramuscularly 40 mg. of neutral red dissolved in 1 or 2 ml. of sterile distilled water. If used in conjunction with histamine stimulation, the injection is made at the same time the histamine is given. When samples are aspirated fractionally, each is examined for the dye which appears in acid medium as a watery pink to red color. If no free acid is present the sample should be acidified to bring out the dye color. The presence of dye in the absence of acid secretion probably indicates a stomach capable of excreting acid and that the achlorhydria is temporary.

EXAMINATION OF STOMACH CONTENTS

Physical Characteristics

The appearance of fasting stomach contents is that of a clear grayish or slightly bile stained fluid containing varying amounts of mucus. Rarely streaks of bright red blood from trauma may be present. Food particles, if present, are an abnormal finding indicative of stasis and probably the result of some obstructive lesion in or near the pylorus. Blood imparts a brownish-red or coffee color.

Volume. The fasting specimen may vary from a few ml. to 50 ml. with an average of about 30 ml. Amounts over 50 ml. are regarded as abnormal. Following an Ewald test meal 20 to 60 ml. are generally present but amounts up to 120 ml. are not regarded as pathologic. After

alcohol test meal or histamine stimulation the total volume of scretion during the ensuing hour varies from 45 to 150 ml.

Color. As indicated, normal gastric fluid is colorless, pale gray, or yellowish to greenish if bile-stained. The same is true of specimens following the alcohol test meal or histamine stimulation. Following an Ewald test meal the fluid is opaque gray. A reddish-brown or coffee color signifies blood.

Mucus. The normal quantity of mucus varies considerably, but large quantities signify a catarrhal type gastritis if contamination by mucus from the mouth or nasopharynx is excluded. It appears as a slimy, stringy, viscid material, generally clear and translucent.

Odor. Normal gastric fluid is odorless or may possess a slightly sour odor. Infected or necrotic lesions impart a foul odor and a high intestinal obstruction a fecal odor.

Food Particles. Any recognizable food particles from a previous meal indicate some degree of obstruction. A large volume of the fasting specimen plus food particles indicates stasis and probably some obstructive lesion at or near the pylorus.

Chemical Examination

Chemical examination of gastric contents, both fasting and after test meals, is the most significant portion of a gastric analysis. If the specimen is not clear, contains much mucus, food, or particles from an Ewald test meal, the specimen should be filtered through a double layer of coarse gauze. Ordinarily this is not necessary on specimens collected following the alcohol meal or histamine stimulation. Tests for free hydrochloric acid, total acidity and blood are of primary importance and are performed routinely while those for lactic acid, pepsin, and rennin generally are done only with specific indication.

Acid. The acidity of gastric juice normally is due to the secretion of hydrochloric acid by the parietal cells of the mucosa. A portion of the acid combines with proteins or protein-like substances to form protein salts of hydrochloric acid which are termed the combined acid. The acid in excess of this remains free and is called free hydrochloric acid.

The concentration of free hydrochloric acid in the gastric juice varies in the fasting specimen and with the type of stimulation used. Providing the stomach is capable of responding to stimulation, the amount of acid secreted generally corresponds to the strength of the stimulus, with the Ewald meal being least, and histamine most, effective.

A wide range of variation in the concentration of acid with any single

type of stimulation may be observed in individuals who are apparently normal. In such a group, using the alcohol meal, Townsend found concentrations of free acid varying from 0 to 97 degrees with an average of 50 degrees. With histamine stimulation higher concentrations have been observed ranging from 0 to 160 degrees with the majority falling

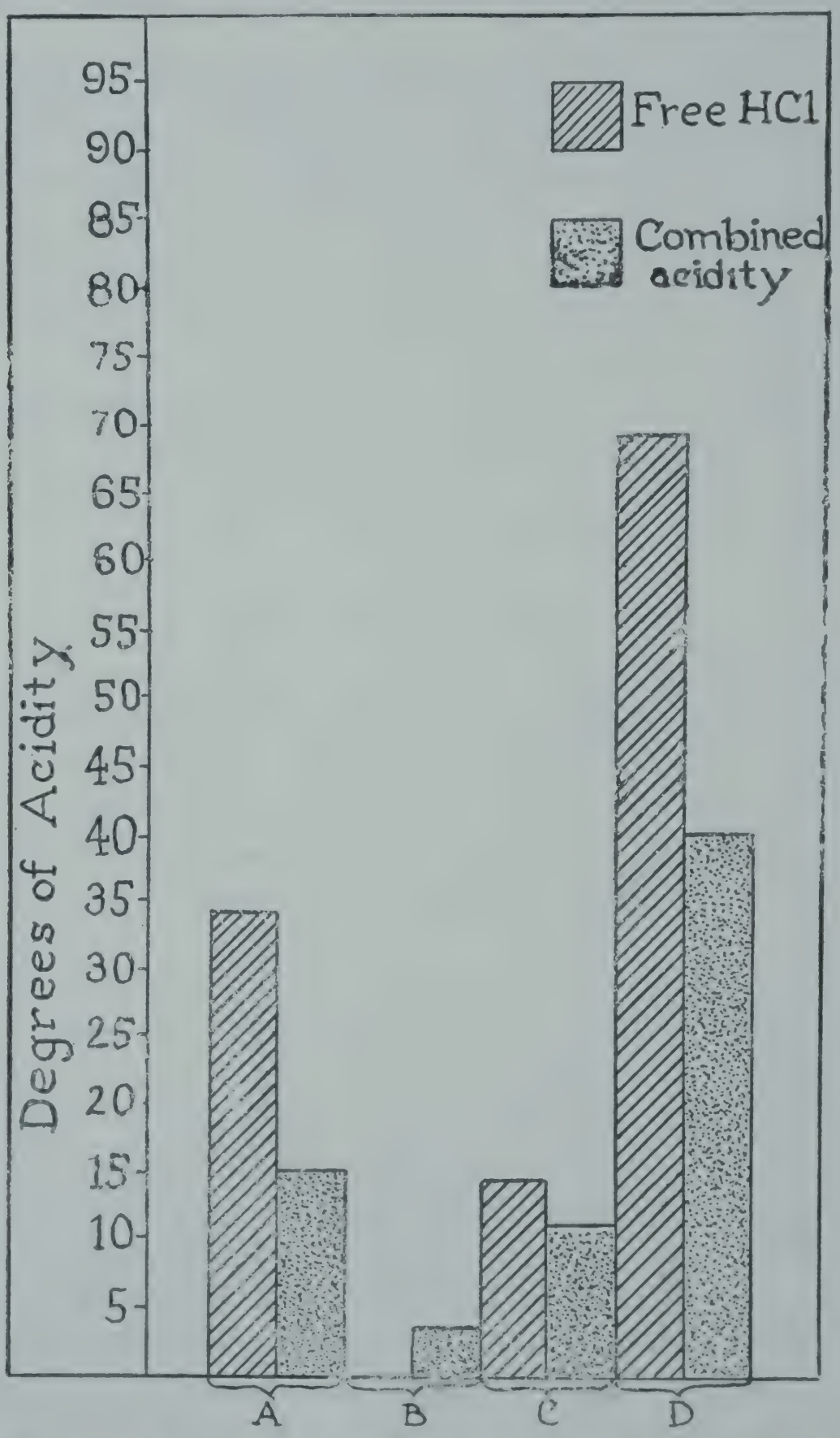


FIG. 23.3. Diagram showing the average amounts of free HCl and combined acidity with the Ewald meal in: A. The normal person. B. Achlorhydria as in pernicious anemia. C. Hypochlorhydria as seen in achlorhydric microcytic anemia. D. Hyperchlorhydria as seen in duodenal ulcer.

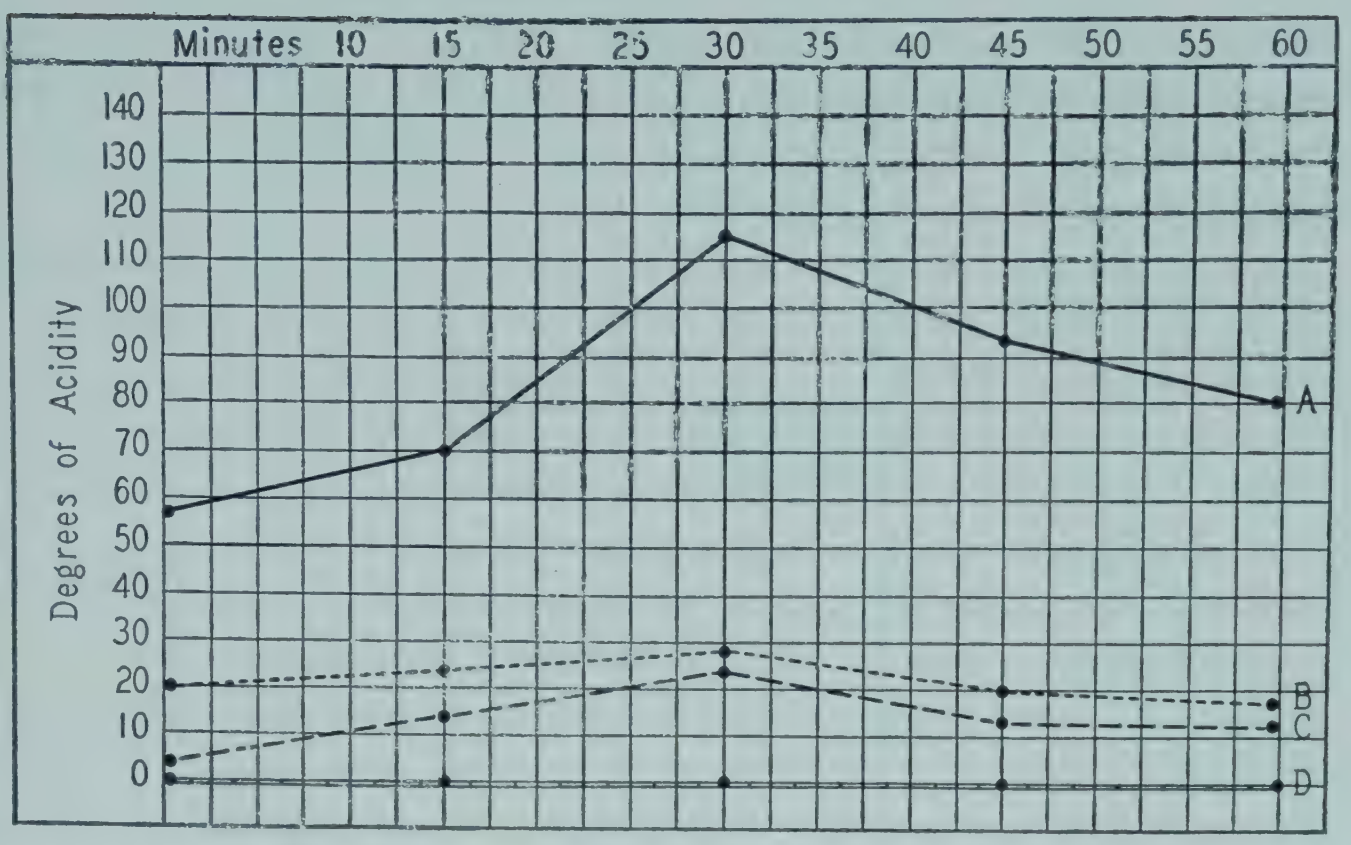


FIG. 23.4. Curves obtained for free HCl in fractional gastric analysis with the alcohol meal. Specimens taken in the beginning, at 15 minutes, 30 minutes, and at 60 minutes. A. In hyperchlorhydria. B and C. In hypochlorhydria. D. In achlorhydria.

between 80 and 130 degrees. The Ewald meal elicits a response of between 30 and 50 degrees.

When the results of fractional analysis are plotted as a graph, the peak of acid secretion is usually found to occur between 30 and 75 minutes after beginning stimulation. With the alcohol meal this subsides rapidly to fasting level but with histamine the high level may be sustained for periods up to an hour.

Free acid is normally low or absent in infants and average values are slightly higher in men than in women.

Combined acidity also varies greatly with the type of test meal used, the amount under normal conditions being governed by the quantity of protein secreted as well as that contained in the test meal. Thus the lowest concentrations are observed after alcohol, histamine or insulin stimulation and higher values after food. Generally the total acidity will vary from 4 to 20 degrees higher than the level of free acid and as long as the latter is within normal range little additional information is gained by the titration of combined acid.

Abnormal concentrations of free hydrochloric acid are separated into three categories: *hyperchlorhydria*, those with values above the high normal levels, *hypochlorhydria* in which the acid concentration is low but not absent, and *achlorhydria* or absence of free hydrochloric acid.

Hyperchlorhydria, in addition to its occurrence in normal individuals, is seen commonly with duodenal ulcer, frequently with gastric ulcer and occasionally with early gastritis, cholecystitis, cholelithiasis, in neurotic individuals and in heavy smokers.

Hypochlorhydria occurs in such a variety of gastrointestinal conditions as well as in many unrelated diseases that little diagnostic significance can be attributed to it. It is frequently present in carcinoma of the stomach, gastric syphilis, chronic gastritis, combined lateral sclerosis, certain types of microcytic-hypochromic anemias of women, and in normal pregnancy. Individuals with gastric ulcer show low acid concentration in a sufficient number of instances to reduce greatly the diagnostic value of acid determinations in this condition.

Achlorhydria may be divided into false and true types and the latter term should be used to indicate an absence of hydrochloric acid in accordance with the criteria of Winkelstein, i.e., the absence of acid be complete, more or less permanent, with a gastric pH of 3.5 or over. Thus only absence of acid after histamine stimulation may be called true achlorhydria and accepted as significant. The occurrence of *true achlorhydria* indicates the probable presence of organic disease and is specifically associated with pernicious anemia, sprue, pellagra, advanced gastric carcinoma, chronic gastritis, combined lateral sclerosis and exophthalmic goiter. The occurrence in normal individuals is variously reported between 1 and 2 per cent.

Combined acidity may be increased in the presence of lowered or absent free hydrochloric acid and such occurrence indicates the presence of abnormal amounts of organic acids, probably lactic acid.

Tests for Free Acids

Congo-red Paper:

A strip of Congo-red paper moistened with gastric contents containing free acid turns a deep blue color.

Töpfer's Reagent:

Add 1 drop of Töpfer's reagent (See Quantitative Determination of Free Hydrochloric Acid) to 1 ml. of gastric contents. A cherry red color indicates the presence of free acid (Plate I).

Test for Free Hydrochloric Acid

Tests for free acids alone when positive, indicate only a gastric pH of 3.5 or below. For the specific determination of free hydrochloric acid the Boas test may be employed.

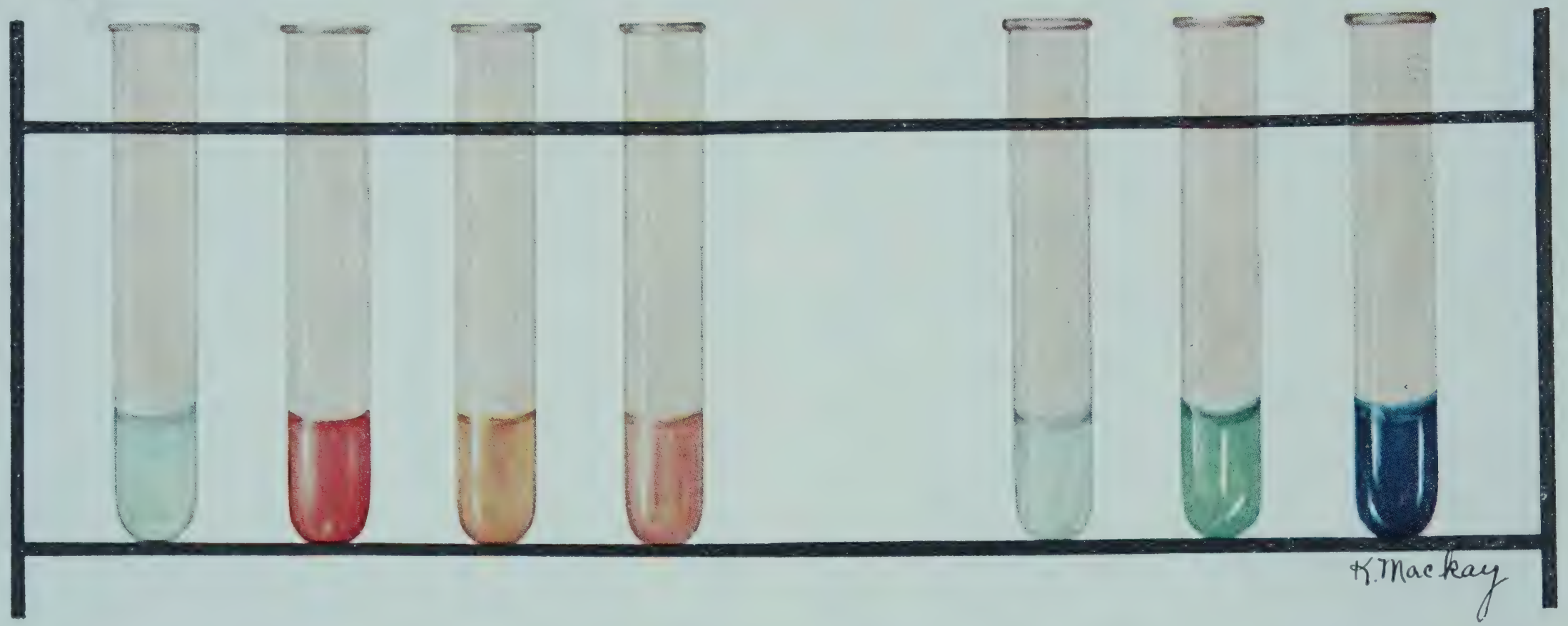


PLATE 23.I. Chemical reactions of gastric juice. Left to right: Tube 1. Clear gastric juice. Tube 2. Red color on addition of dimethylaminoazobenzol (Töpfer's reagent) indicating presence of free hydrochloric acid. Tube 3. Salmon-yellow color, endpoint of titration of free hydrochloric acid with N/10 sodium hydroxide. Tube 4. Pink color, endpoint of titration of combined acid using phenolphthalein as the indicator. Tube 5. Negative benzidine test for blood. Tube 6. Slightly positive benzidine test for blood. Tube 7. Strongly positive benzidine test for blood.

Quantitative Determinations of Free Hydrochloric Acid and Total Acidity

Principle:

The presence of free hydrochloric acid causes the gastric contents to have a pH of 3.5 or less. Neutralization with sodium hydroxide solution is carried out until the end point of Töpfer's reagent (pH 4) is indicated by change from cherry red to salmon pink.

Phenolphthalein is then added and the titration is continued until combined acid is neutralized as indicated by a faint pink end point (pH 9–10). (Plate 23.I)

Procedure:

1. To 5 ml. of clear gastric contents in a small beaker or evaporating dish, add 1 to 2 drops of Töpfer's reagent. A cherry red color indicates the presence of free hydrochloric acid.
2. Add N/10 sodium hydroxide solution from a buret or pipette, drop by drop with stirring, until the color changes to salmon pink. (Yellow is the complete end point but the use of salmon pink prevents over titration.) Observe the amount of alkali used.
3. Add 1 to 2 drops of phenolphthalein solution and continue titration as above until the appearance of the first permanent pink color. Observe the amount of alkali used.

Calculation:

No. ml. N/10 NaOH (1st titration) × 20 = degrees of free HCl
No. ml. N/10 NaOH (both titrations) × 20 = degrees of total acidity.

Note:

1. If the quantity of gastric contents is limited the titration may be performed on smaller amounts of fluid and the calculation altered accordingly.
2. False low values for free acid will be present if the gastric conter ts contain bile or large quantities of mucus, and if the patient who has had histamine stimulation swallows his saliva.

Lactic Acid. Lactic acid is not present in normal gastric juice. It may be introduced into the stomach with certain foods, such as sour milk or food prepared with it. It may be formed in the stomach from fermentation of carbohydrate foods by lactic acid producing organisms, such as Boas-Oppler bacilli, when the emptying of the stomach is delayed or the secretion of hydrochloric acid is low. This occurs chiefly in advanced carcinoma of the stomach.

Enzymes. The enzyme activity of gastric juice is due to the presence of pepsin, rennin, and lipase. Pepsin and rennin are elaborated by the chief cells in the inactive forms, pepsinogen and renninogen which are activated by free hydrochloric acid. There is question regarding the separate identity of these enzymes. They are so constantly associated

with hydrochloric acid in gastric juice that their presence may be assumed with the finding of free hydrochloric acid. However, psychic influences may lower the acid values without affecting the production of enzymes. An absence of pepsin or rennin, therefore, indicates organic disease of the stomach.

Gastric lipase is elaborated in active form and differs from pancreatic lipase only in being active in an acid medium (pH 4–5).

Blood. Blood in the gastric contents may be caused by pathologic lesions of the stomach or esophagus, trauma incidental to the passage of the stomach tube, or to material swallowed. The most common pathologic lesions responsible for hemorrhage are peptic ulcer, malignancy, and esophageal varices. In all of these conditions the blood is usually acted upon by hydrochloric acid producing brown acid hematin. This gives the gastric contents its characteristic coffee color. The presence of bright red blood indicates either very recent hemorrhage or absence of acid.

Blood due to tube trauma generally appears as small, bright red flecks seen as the material is aspirated from the stomach. This finding is of no consequence.

With lesions outside the gastrointestinal tract such as hemorrhage in pulmonary tuberculosis, epistaxis, bleeding from gums, posterior nasopharynx, or from tooth extraction, varying amounts of blood may be swallowed and thus appear to have originated in the stomach. Considerable blood may be swallowed by breast-fed infants in nursing a cracked nipple. Thus, with such a variety of possibilities of origin, a negative test for blood is of more significance than a positive one.

Test for Blood. The benzidine test as used for the detection of blood is performed as for the detection of blood in urine.

DETERMINATION OF ACHLORHYDRIA WITHOUT INTUBATION

The intubation of the stomach is a rather unpleasant experience for many individuals; since frequently the principal information desired is a determination of achlorhydria, a method of determining this point without intubation is of real importance. Segal, in 1950, and numerous workers since, have amply demonstrated that this is possible. The test is carried out by the ingestion of a guininium resin indicator and subsequent examination of the urine for quinine. It is based on the free hydrogen cations of dilute hydrochloric acid in the gastric juice, readily displacing the quininium ion of the resin indicator. The released quinine is absorbed in the small intestine and about one third excreted in the

urine. The test is carried out as follows:

1. No drugs on day preceding test, particularly quinine or related drugs, B-complex vitamins, or steroid compounds.
2. No food after midnight.
3. Pass urine on arising; discard this urine.
4. No breakfast. Empty contents of capsule in small packet (250 mg. of caffeine sodium benzoate) into glass of water, stir and drink. Drink another $\frac{1}{2}$ glass of water or cup of coffee or tea, without cream, milk or sugar.
5. One hour later, or as soon after the 1-hour interval as possible, urinate and save this urine (entire quantity) in bottle labeled "Urine Control."
6. Empty the granules of quininium resin in the large packet into a $\frac{1}{4}$ glass of water, stir well and drink. (The granules do not dissolve; do not chew them.) Fill glass again $\frac{1}{4}$ with water, rinse mouth and swallow.
7. After 2 hours urinate and save entire quantity in bottle labeled "Urine Sample No. 1."

The resin indicator is marketed under the name Diagnex. The first 2-hour urine specimen after administration is the important one for the determination of free hydrochloric acid in the stomach. Quinine found in later urine specimens may not be significant of free acid in the stomach.

In the laboratory the 2-hour urine specimen is diluted to 300 ml. with distilled water. A 30-ml. portion is then made alkaline with 0.5 ml. N/2 sodium hydroxide and extracted with 15 ml. of ether. 8.2 ml. of the ether extract is treated with 5 ml. N/10 sulfuric acid and this solution examined for fluorescence under ultraviolet light using the control sample. A semi-quantitative examination may be made by preparing standard solutions of quinine. By this technique free hydrochloric acid is present in the stomach if more than 25 micrograms of quinine are found in the urine sample. Readings of less than 15 micrograms are reported negative and from 15 to 25 micrograms as a trace.

This tubeless method is safe and highly accurate when compared with analysis secured by the tube method. However, there is no apparent quantitative relationship between the two methods for high excretion of quinine has been found with low acid values and vice versa.

MICROSCOPIC EXAMINATION

Microscopic examination should be performed on the sediment from a centrifuged specimen of the fasting contents before a test meal is

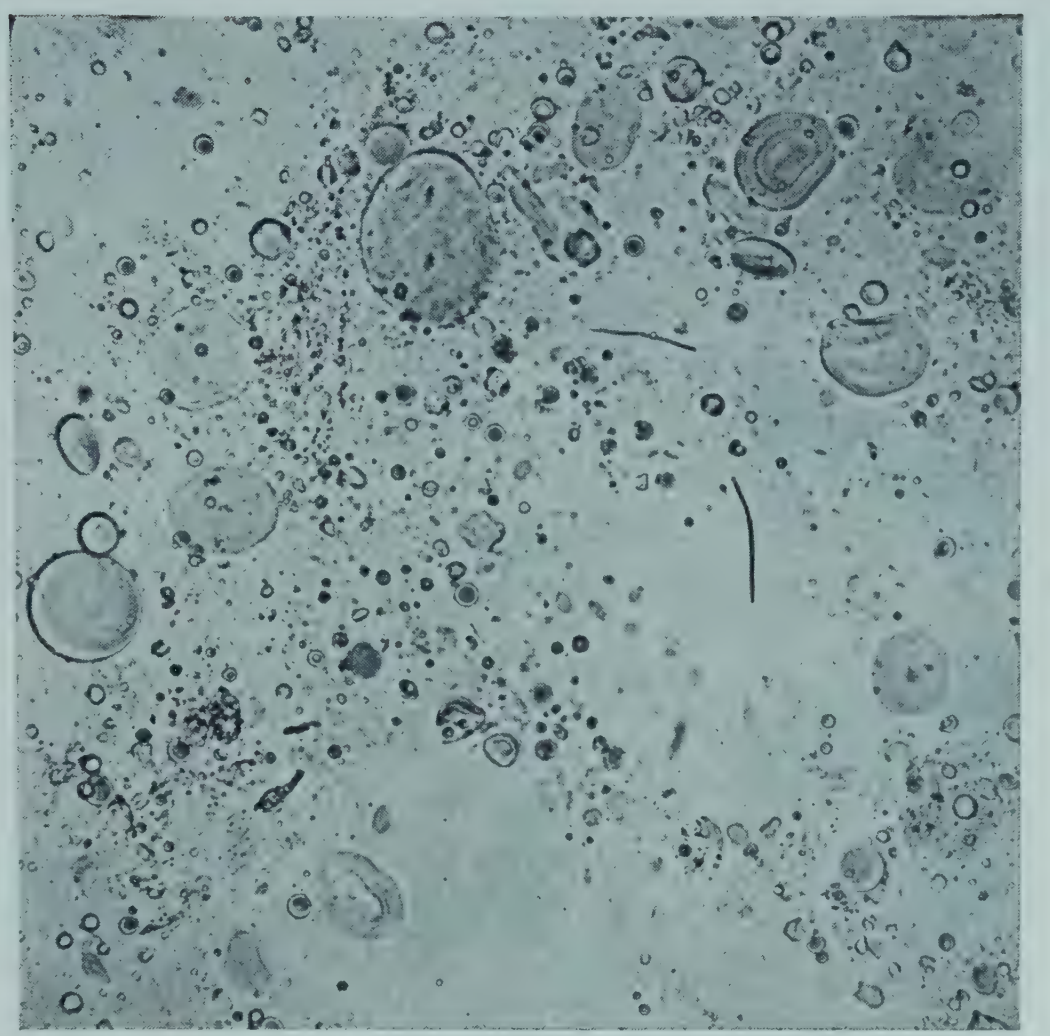


FIG. 23.5. The sediment of gastric contents after a bread and water test meal. Note the starch granules, yeast cells and a few large bacilli that are identical with Boas-Oppler bacilli.

given as there is less dilution of any formed elements which may be present. If this specimen is unobtainable or insufficient in amount, contents removed after stimulation may be used. Cover slip preparations are made and examined unstained with low and high dry magnification. Preparations stained with Giemsa's, Gram's and acid fast technics are of value for identifying cells, tissue and bacteria. Wet preparations to which is added a drop of Lugol's solution are helpful in differentiating starch granules from yeast cells, the former staining blue or reddish depending upon degree of digestion and the latter yellow.

Normal gastric sediment may contain starch granules, yeast cells, epithelial cells, a few fat globules and bacteria.

Pathologically the sediment may contain muscle fibers, red blood cells, pus cells, fragments of tissue and large numbers of bacteria.

Muscle fibers, best recognized in unstained preparations by the presence of striations, indicate stasis.

Red blood cells, in the presence of free hydrochloric acid, are usually hemolyzed unless the quantity of blood is great or the hemorrhage quite recent. Incompletely destroyed cells may show as granule clumps.

Small numbers of well preserved red cells in a relatively clear medium suggest trauma or swallowed blood.

Pus cells are uncommonly encountered and when present are frequently the result of swallowing material from the respiratory tract or the sinuses. They may be in their usual form or when partially digested show as clusters of nuclear material. They are best demonstrated in Giemsa stained preparations.

Bacteria most commonly seen are Boas-Oppler bacilli, large Gram positive bacilli in conditions associated with stasis and low acidity. Sarcinae, large coccoid organisms arranged in cuboidal groups, are commonly found in conditions associated with stasis and high acidity as in duodenal ulcer.

Culture of the gastric contents by Dick has shown that acid stomachs are practically free of bacteria. In pernicious anemia the flora were found to be quite distinctive, containing large numbers of organisms prominent among which were *Streptococcus viridans*, members of the colon group, and Gram-negative threadlike anaerobes. In contrast, the gastric contents from carcinomatous stomachs showed only moderate numbers of organisms, including *Streptococcus viridans* and Boas-Oppler bacilli but no colon organisms or threadlike anaerobes.

Tubercle bacilli are present with great frequency in the gastric content of all individuals with pulmonary tuberculosis, but particularly in women and children, who have a great tendency to swallow rather than expectorate sputum. Routine acid-fast examinations of the gastric sediment of individuals suspected of having pulmonary tuberculosis, on whom there had been repeatedly negative sputum examinations, yielded positive findings in 20 to 30 per cent of cases. Guinea-pig inoculation of the gastric contents is even more delicate, yielding additional positive results.

Tissue fragments may be present with carcinoma of the stomach and the specific diagnosis of cancer can be made on Giemsa-stained preparations. Suspicious fragments seen grossly should be pressed between glass slides, smeared, and treated as an aspiration biopsy.

The total fasting gastric specimen may be centrifuged and the sediment fixed with twice its volume of 95 per cent alcohol and again centrifuged. This sediment is spread on glass slides and smears made. If cancer is strongly suspected, following the aspiration of the fasting content the patient is given 8 ounces of Ringer's solution by mouth, a 50-ml. syringe attached to the tube, and the fluid withdrawn and reinjected several times. The washings thus obtained are centrifuged and the sediment fixed by twice its volume of 95 per cent alcohol.

Gentle mechanical irritation by use of a balloon or by a gastric brush has proven highly successful in the hands of skilled operators. The balloon or brush is slowly moved up and down to cover as much of the entire surface of the stomach as possible 5 or 6 times. When using the balloon all accumulated fluid is aspirated several times during this period of irritation. The aspirated material is centrifuged, the sediment fixed with twice its volume of 95 per cent ethyl alcohol, and smears made. The balloon brush is washed, the fluid centrifuged and the sediment treated the same way. The smears can be stained with Giemsa stain for 20 to 40 minutes, then washed with buffered water, dried, and examined under oil immersion for malignant cells.

Duodenal Contents

The fasting duodenal contents usually amount to about 20 ml. and consist of a mixture of pancreatic and duodenal secretions, bile, and variable amounts of gastric contents. Examination of this fluid as well as that aspirated after stimulation by magnesium sulfate or olive oil may yield valuable information concerning the functional state of the gallbladder, biliary passages, and pancreas; the patency of the pancreatic duct; and the presence or absence of inflammatory lesions in the duodenum.

COLLECTION OF DUODENAL CONTENTS

Either the Levin or the Rehfuss tubes may be used for the collection of duodenal contents. The latter is recommended since the metal tip facilitates more rapid passage from the stomach into the duodenum. The tube is passed as previously described (the patient having fasted for 12 hours) until the last mark is at the teeth, indicating the insertion of sufficient tube to allow passage into the duodenum. Have the patient lie on the right side with the hips slightly elevated, which position permits gravity to aid peristalsis in carrying the tube through the pylorus. This is usually accomplished within 30 to 45 minutes and is indicated by an alkaline reaction of the aspirated fluid. The fluid obtained in this fashion is that secreted without stimulation.

If examination of biliary contents is desired, 50 ml. of sterile 25 per cent magnesium sulfate solution are injected through the tube. This produces relaxation of the sphincter of Oddi with resulting outpouring of bile which may be collected by aspiration.

Bile is primarily from the common, cystic and hepatic ducts and is light in color. Bile from the gallbladder frequently may be collected more satisfactorily after olive oil stimulation. A mixture of 15 to 30 ml.

of olive oil and 15 ml. of hot water is introduced through the tube and after a few minutes the contents are aspirated. This bile is usually dark in color. If examination is directed primarily toward analysis of pancreatic juice, a cup of bouillon should be given about one-half hour before the tube is inserted.

EXAMINATION OF CONTENTS

The fluid collected should be centrifuged, the supernatant portion decanted for chemical examination and the sediment saved for microscopic study. Examination should be performed within 30 minutes after collection.

Physical Characteristics

Normal duodenal content is a slightly syrupy, water-clear or pale yellow fluid which is alkaline in reaction. It may be discolored by bile, blood or pus, or be opaque from admixture of gastric juice or the presence of undue amounts of crystals, bacteria, and cells.

Chemical Examination

Reaction. Normally the reaction is alkaline to litmus paper but, if mixed with sufficient gastric juice, may be acid and unsuitable for proper examination.

Urobilin. Small amounts of urobilin are present normally. Increased amounts may be determined quantitatively, and, as in the case of urobilinogen, indicate increased blood destruction. Since the same information may be obtained by estimation of urobilinogen in urine, this test is recommended.

Enzymes. Analysis of the enzyme content of the duodenal fluid is infrequently called for, having been replaced largely by determination of blood amylase (See Chapter 7 on Blood Chemistry).

Microscopic Examination

Place a portion of the sediment from the centrifuged specimen on a slide and cover with cover slip. Examine with reduced light. Cells that may be present are epithelium from duodenum or biliary passages, leukocytes or red blood cells.

Cholesterol crystals may be present and either appear as splinters of glass or less frequently take the more characteristic form of squares or rectangles with one corner cut out. Golden yellow amorphous calcium

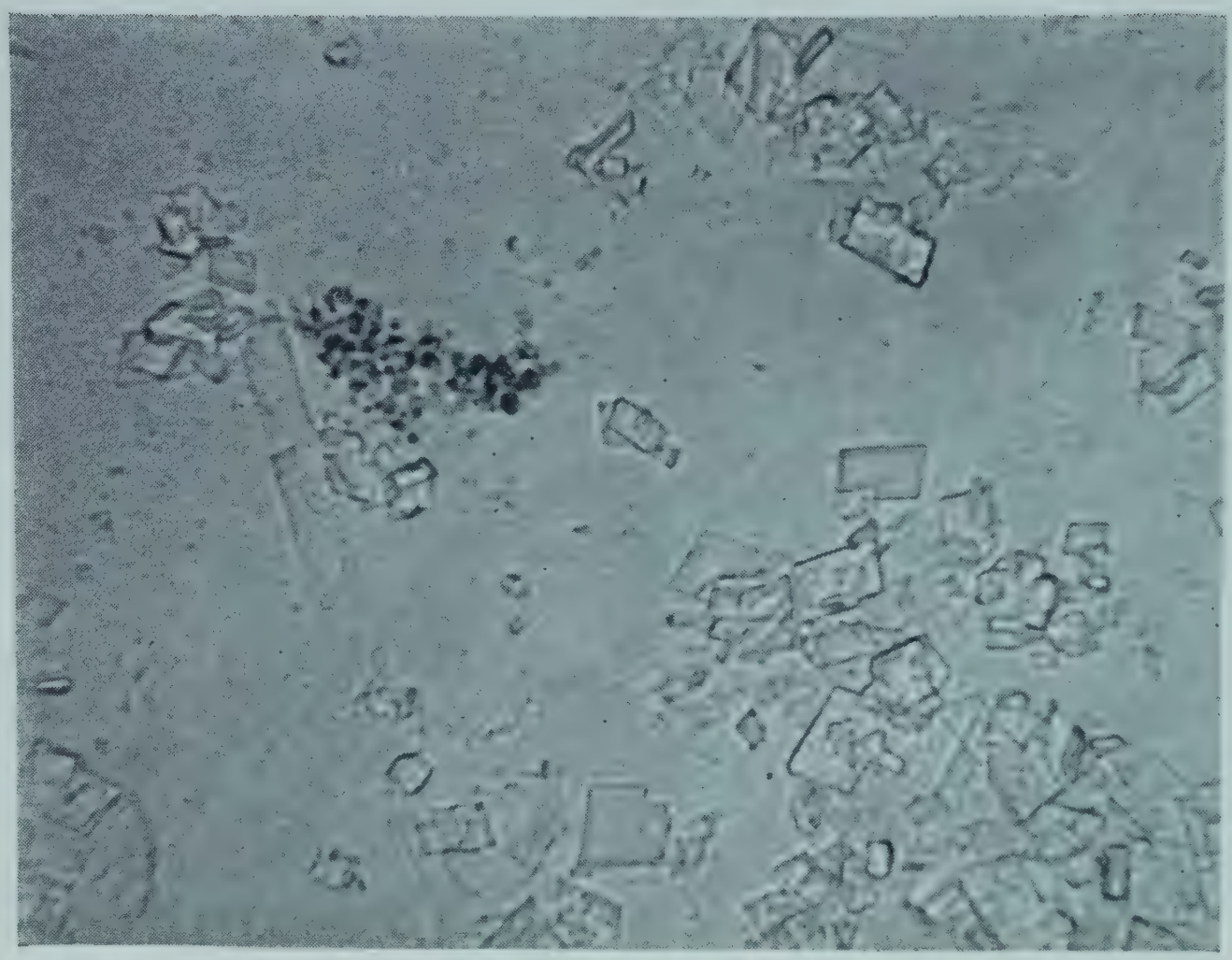


FIG. 23.6. Cholesterol crystals in duodenal contents. Upper left shows a clump of biliary pigment and desquamated cells. Courtesy of Dr. H. C. Myers.

bilirubin may be identified and when occurring with cholesterol crystals is strongly indicative of biliary calculi (Fig. 23.6).

Larval forms of *Strongyloides stercoralis* and *Giardia lamblia*, the ova of *Necator americanus* and cysts of *Endamoeba histolytica* have been observed. Bacteriologic studies are of little clinical value.

Interpretation of Gastric and Duodenal Analysis

Normal findings may vary considerably, particularly in the amount of fasting gastric contents and its acidity. Likewise, the amount of mucus present in normal individuals varies greatly. Microscopic examination of a sediment of the fasting specimen should never be omitted. The identification of many food particles indicates some stasis and probable obstruction. A few blood cells are meaningless but numerous blood cells indicate probable hemorrhage from a varix or ulcerative process. Pus cells and bacteria are not significant of any specific gastric pathology and indeed when present are most frequently from sinus; nasal or oral pathology. Tubercle bacilli are commonly present in the fasting gastric content of cases of pulmonary tuberculosis due to nocturnal swallowing of the sputum.

The absence or presence of free hydrochloric acid is the principal objective of most gastric analyses. Free hydrochloric acid usually varies from 30–50. This decreases with age and a significant number of otherwise normal individuals show no free hydrochloric acid in their fasting gastric contents. More and more, histamine is being used to stimulate gastric secretion; indeed true achlorhydria exists only when free hydrochloric acid is absent after adequate stimulation by histamine. The alcohol and other test meals are not adequate to establish this vital point. True achlorhydria is found practically only in pernicious anemia and subacute combined degeneration of the spinal cord. In fact, it is doubtful if the diagnosis of pernicious anemia can be made without a true achlorhydria.

Duodenal contents are examined principally for the presence of bile and establishing the presence of dark colored bile from the gallbladder is frequently important. Pancreatic enzymes can be measured in the duodenal contents but are less reliable than blood levels. Again microscopic examination should always be made the finding of parasites, ova or cysts are obviously significant while finding amorphous calcium bilirubin and cholesterol crystals is suggestive of biliary calculi.

As with many other laboratory findings, the results of gastric or duodenal analysis should not be considered alone. The information so gained should be evaluated along with the patient's symptoms and other pertinent findings such as hematologic, neurologic, and roentgenologic. Only then can these findings be most meaningful and helpful in establishing the diagnosis.

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CHAPTER 24

SEMINAL FLUID

By Seward E. Miller

GENERAL CONSIDERATIONS

In about 40 per cent of infertile couples the husband is at fault. Because of greater simplicity a careful appraisal should be made of the husband's fertility prior to exhaustive studies of the wife. Examination of the semen is the most critical portion of this appraisal. In addition, examination of the seminal fluid is necessary in checking on the effectiveness of a previous vasectomy and finally in medicolegal cases when paternity is disclaimed on the basis of sterility.

Recently it has been demonstrated that seminal fluid may be quick frozen and stored at −70°C. until needed for insemination. Successful inseminations have been effected using previously frozen seminal fluid. This obviously may create most intriguing and vastly potent possible family situations.

The laboratory analysis of semen consists of the following:

Examination	*Normal Values*
1. Liquefaction	Complete within 15 minutes
2. Volume	2–5 ml.; average 3–4
3. pH	7.2–8.0; average 7.8
4. Leukocytes	Only an occasional
5. Count	60 to 150,000,000; below 60,000,000 abnormal
6. Motility	80 per cent or more vigorously active; less than 80 per cent abnormal
7. Morphology	80–90 per cent normal forms; less than 80 per cent normal forms is abnormal

PRODUCTION OF SEMINAL FLUID

The convoluted portion of the seminiferous tubules of the testis is lined by several layers of germinal epithelial cells. After puberty these cells are constantly producing spermatozoa. The immature forms from beneath become spermatocytes and later spermatids which develop into the mature spermatozoa. These traverse the epididymis, the vas deferens, and ejaculatory ducts which unite to form the seminal vesicles.

The many glands along this entire tract excrete fluid which, mixed with the prostatic fluid, forms the vehicle for transporting and protecting the spermatozoa. The semen or seminal fluid is a mixture of all these

secretions and spermatozoa. However, there is considerable variation in composition between different portions of the fluid as ejaculated. The first portion consists of the excretion of the urethral glands plus mostly prostatic secretion and few spermatozoa. The mid portion contains the bulk of the spermatozoa while the last portion consists chiefly of seminal vesicle secretion with few spermatozoa. This fact is utilized in selecting the mid portion for use in artificial insemination.

COLLECTION OF THE SPECIMEN

Three methods are in common use: (1) Self-produced specimen, collected at the office or laboratory, (2) Emission, after interrupted coitus, into a clean wide mouth glass bottle, and (3) Condom collection. The condom first must be washed thoroughly to remove powder and then dried. After coitus, the condom is tied, wrapped in a clean handkerchief and brought to the laboratory.

All specimens collected outside should be brought to the laboratory as soon as possible, preferably in 30 minutes, but never over two hours. Whenever seminal fluid is collected outside and brought in for examination it must be kept properly warm. The correct temperature may be achieved by carrying the specimen strapped against the skin beneath the clothing. A greater degree of heat should never be applied.

PHYSICAL CHARACTERISTICS

The freshly ejaculated specimen consists of an opaque grayish to yellowish semigelatinous mass of high viscosity. The amount varies greatly from a few drops to 8 or 10 ml. The average is 3 to 4 ml. It is alkaline with a pH of 7.2 to 8.0, with an average of 7.8. It may be odorless or have a slight musty odor. Liquefaction occurs rapidly and is complete in about 15 minutes. An occasional leukocyte, epithelial cell, or crystal, is seen; rarely red blood cells and bacteria are present.

MICROSCOPIC EXAMINATION

Sperm Count

Using a white blood cell pipette the count is made as for white blood cells except the diluent is composed of 5 per cent sodium bicarbonate and 1 per cent phenol in distilled water. Two or three counts from the well mixed specimen should be made and the average used. The normal count has a wide variation between 60,000,000 and 150,000,000 per ml., although counts as high as 300,000,000 per ml. are occasionally encountered.

Motility

In an initial thick smear preparation of normal semen about 80 per cent of the spermatozoa should be actively motile with about 20 per cent sluggish or non-motile. *Azoospermia* is the complete absence of sperm, while in *necrospermia*, although sperm are present, they are completely immobile. In *oligozoospermia* only a few motile sperm are present. Azoospermia is due to bilateral small under-developed testes or obstruction from previous traumatic or operative procedures, with gonorrhea now a secondary cause. Necrospermia never occurs with a normal count but is always seen in cases with bilateral hypotrophic testes. Oligospermia may be due to bilateral or even unilateral hypotrophic testis or hypothyroidism, but more frequently no cause can be discovered.

Hourly preparations should be made and observed for motility. At the end of six hours there should be a goodly number of actively motile forms with about a 10 per cent loss in motility each previous hour.

Morphology

The normal sperm consists of a relatively large oval head, a small neck and connecting piece with a long slender tail. The entire structure meansures 50 to 70 microns in length, with the tail composing 90 per cent. The oval head is also flattened and is 3 to 6 microns long by 2 to 3 microns wide. The base of the neck is connected to the axial filament, running backward down the tail which produces the active motion.

Abnormal forms are studied best on a stain smear. Smears are prepared and air-dried as blood films. The mucus is removed by flooding with the phenol-sodium bicarbonate diluting fluid, washed with buffered water and stained with Giemsa stain 20 to 40 minutes. Normally, the sperm heads are quite uniform in size and shape. Abnormalities to be looked for are excessive tapering or round heads, also "giant" heads and "pin" heads, and finally duplicate heads. The head stains lightly anteriorly and more deeply posteriorly. Any deviation in size, shape, or staining reaction is regarded as abnormal. Coiling or bending of the tails is of no significance.

Interpretation

The specimen is collected preferably in the laboratory in a clean, small, wide-mouth bottle after abstinence from intercourse for 4 or 5 days. The volume and pH may be immediately determined and the specimen observed for completion of liquefaction which occurs in about

15 minutes. Observation for liquefaction is important, for if liquefaction does not occur normally motility is seriously interfered with. After liquefaction is complete the cell count, motility and stained smears for morphology studies can be made. Color, odor, and pH are normally of no significance, while the volume, cell count, motility, and morphology are the important factors.

There appears to be some normal variation in specimens from the same patient. Particularly is this apt to be true of the sperm count. Therefore, in borderline and even in frankly abnormal cases several specimens should be examined and the one with the highest values used.

While in general the per cent of abnormal forms and loss of motility increase proportionately as the sperm count decreases, still examination for motility and abnormal forms cannot be omitted because of the infrequent case in which this is not true. Generally, however, the sperm count is a good index of fertility level. Necrospermia apparently never occurs with a count above 60,000,000.

An as yet unexplained phenomenon of agglutination has been observed rarely and in this condition all motility is lost after 15 minutes dispite a normal count and an otherwise normal specimen.

There is as yet no general agreement as to the practical significance of these various factors. Several arbitrary standards for normal semen have been proposed. The American Society for the Study of Sterility has proposed a volume of 2–5 ml., a minimum count of 60,000,000 sperm per ml., at least 60 per cent vigorously active forms after liquefaction, and not over 25 per cent abnormal forms.

Fertility appears to diminish rather rapidly with a density of less than 60,000,000 sperm per ml., although rarely pregnancy does occur with rather low counts. The percentage of actively motile sperm and the percentage of abnormal forms both assume greater significance with counts of 60,000,000 per ml. or less.

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CHAPTER 25

FECES

By Seward E. Miller

GENERAL CONSIDERATIONS

Examination of the feces may reveal the presence of a specific disease agent such as an intestinal parasite or indirect evidence of malfunction of some portion of the gastro-intestinal tract, the liver, or pancreas. The full significance of fecal examinations generally is overlooked by both the clinician and the laboratory. Even when alert clinicians request various examinations of the feces, too frequently these important procedures are delegated in the laboratory to an inexperienced or poorly trained individual. Fecal examination in the average clinical laboratory is generally perfunctory and almost useless despite the well-established fact that there is a wealth of clinical leads inherent in intelligent fecal examinations.

Normal Findings

The normal stool consists chiefly of undigested food remnants, water, various products of digestion and decomposition, and enormous numbers of bacteria—most, if not all, of which are non-pathogenic. In fact, a significantly large part of the total volume of feces is made up of bacteria, dead or living. Food remnants are greatly increased in diseases in which digestion is impaired and in those accompanied by diarrhea, when food is swept onward before digestion and absorption are complete. Many of these food remnants can be recognized and identified either macroscopically, such as seeds, fruit or vegetable skins, etc., or microscopically, such as muscle fibers, vegetable cells, fat droplets, hairs, etc.

COLLECTION OF SPECIMENS

Fecal specimens should be passed in a bed pan without admixture with urine and should be made available to the physician or laboratory worker at once for inspection and examination. If this cannot be done careful inquiry should be made as to the appearance of the stools and the patient or attendant should be carefully instructed as to the amount desired and should be furnished with a suitable container. For hospital practice, a 4- or 8-ounce ice cream carton is most suitable; for office practice, a 2- or 4-ounce wide-mouth bottle with a screw cap. About 10 ml. is

sufficient quantity except for some special examinations. Sufficient feces for microscopic examinations and bacterial culture will adhere to a finger cot and thus may be obtained when doing rectal examinations.

When the fecal specimen must be shipped to a distant laboratory for bacteriological examination, a preservative such as buffered glycerol-saline should be used. In suspected cases of amebiasis a fresh warm stool specimen is imperative, for *E. histolytica* trophozoites disintegrate quite rapidly and their characteristic ameboid movements cease unless the stool is kept warm. However, an excellent preservative for *E. histolytica* trophozoites and cysts has been developed: polyvinyl alcohol. (For further discussion, see Chapter 14, Medical Parasitology, Section on Amebiasis.) When a stool must be shipped and an examination for amebiasis is desired, it is imperative to use polyvinyl alcohol as a preservative.

MACROSCOPIC EXAMINATION

Much evidence of malfunction of the gastro-intestinal tract may be obtained by careful inspection of the fecal specimen, noting its form and consistency and the presence of mucus, blood, purulent material, particles of undigested food, gallstones, foreign bodies, intestinal parasites, color, and odor.

Form and Amount

Diet and intestinal motility exert the major influences on the form, shape, and consistency of the feces, with a rather wide range of normal. Most adults pass one or two formed stools daily, a few to several inches in length, usually smooth but frequently indented or irregular. However, about 8 to 10 per cent of normal individuals may have bowel movements only every other day or average only three per week. The average daily amount by weight is roughly 200 grams. Predominantly vegetable diets greatly increase the amount of unabsorbed material, resulting in large, soft, thick, nonformed stools, while diets high in protein and carbohydrates with less residue normally result in much smaller, firmer formed stools. Thus, the individual's established bowel habit is exceedingly important in determining the abnormal.

Constipation produces firm or hard stools, frequently grooved or segmented. More intense constipation causes oval or ball-shaped stools of varying size or the pipe-stem or ribbon-shaped stools. These latter forms also occur in stricture of the rectum. The amount of water present in normal adult feces on a mixed diet is about 65 per cent. The

water content largely determines consistency—the more water present the softer and more bulky the stool. Increased motility, particularly in the colonic tract, may result in completely liquid or watery stools so characteristic of diarrhea.

A motility or initial rate of about 14 hours results in soft formless stools, while an initial rate of around 25 hours results in well-formed stools. Initial rates are determined by the time from ingestion of a test substance such as millet seeds until its initial appearance in the stool.

Odor

The characteristic fecal odor is due chiefly to the presence of indole and skatole. Butyric acid, hydrogen sulfide, and methane may also be present. The intensity of the odor depends upon putrefactive bacterial activity and the amount of meat protein. Stools of vegetarians have a less pungent odor, while a strict milk diet may result in stools with quite nonoffensive odor. The fairly characteristic sour odor of infant stools, particularly those with some diarrhea, results from fatty acids. Severe diarrheas of children and adults produce a foul or putrid odor due to putrifaction of undigested food. Unabsorbed meat protein reaching the colon is attached by the putrifying bacteria, and the resulting putrification is responsible for a most offensive putrid odor. Extensive ulcerations and malignant lesions in the lower colon and rectum also may impart a very foul offensive odor.

Color

The brownish color of feces is derived from the bile pigment, bilirubin, which the intestinal bacteria convert to hydrobilirubin, stercobilin. However, diet, drugs, and hemorrhage may influence the color greatly. Chocolate and cocoa impart a gray color; starchy foods and milk, a yellow color; leafy vegetables, a greenish color from the chlorophyll; fats produce the so-called "clay color." Large amounts of unabsorbed fats impart a greasy appearance. The absence of bile pigments from either obstruction or deficient secretion results in the so-called "putty-colored" or "*alcoholic*" stool, while the ingestion of barium imparts a whitish color. Hemorrhage from high in the gastro-intestinal tract produces tar-colored stools, and iron medications a greenish-black to deep black color. Small amounts of blood that escape detection by inspection are termed *occult* blood, which may be detected by chemical

means. Red streaks of blood on the surface of stools come from lesions in the lower colon or rectum.

Mucus

The amount of mucus in normal feces is very small and cannot be recognized by gross inspection. Visible and frequently large amounts of mucus are present in the infectious dysenteries, amebiasis, mucus colitis, intussusception, infected diverticula, and irritative lesions. When the mucus is quite thoroughly mixed with the feces, the lesion is most likely in the small intestinal tract, while mucus present on the surface, especially when mixed with freshly colored blood, is indicative of a lesion in the colonic tract. In mucus colitis, mucus casts of the intestines, most frequently seen after enemas, are sometimes formed, as are irregularly segmented strands and shreds which have been mistaken for intestinal parasites, particularly tapeworms.

Gallstones and Foreign Bodies

Careful inspection of the feces may reveal typical gallstones and all doubtful concretions should be examined chemically. True enteroliths are rare and sandlike grains are usually seeds.

Most foreign bodies that are swallowed and pass through the esophagus will eventually pass through the entire gastro-intestinal tract and appear in the feces, as have amazing numbers of foreign bodies, from small metallic shot, tacks, buttons, and coins to slender, sharp objects such as needles.

Undigested Food

Undigested food remnants frequently are identified—particularly bits of muscle, bone or cartilage, or vegetable particles, such as whole kernels of corn, many raw vegetables, and, of course, seeds and particles of fruits, particularly skin. Starchy foods have no visible residue. The diagnostic significance of undigested food particles often is difficult to determine, for much depends upon the cooking and mastication of the food as well as the digestive process and motility of the gastro-intestinal tract. However, physiological factors being normal with a normal diet, visibly recognizable food particles are rare indeed in the feces.

Parasites

The intestinal tinea parasites, "tapeworms," can be identified in the stool by inspection. When looking for the head it is well to use a hand

lens. The hand lens, too, is needed to identify smaller worms such as the male pinworm. Insect larvae, "maggots," have been reported from the ingestion of food badly contaminated with fly eggs or larvae.

CHEMICAL EXAMINATION

Although the chemical composition of feces is altered in numerous disease conditions, laboratory evidence of such dysfunction generally is obtained by examination of other materials, stomach or duodenal contents, blood, or urine. There remain, however, a few types of chemical examinations that frequently are fruitful—chemical examinations for blood, bile pigments, and trypsin.

Blood

Blood originating from lesions in the stomach and small intestines may be so altered by the digestive processes that it cannot be identified macroscopically. Occult blood may be detected chemically. However, the techniques are so sensitive that for a positive report to be significant of a possible gastro-intestinal tract lesion the patient must have been on a meat-free diet for at least three days preceding the test, and swallowed blood from the mouth, gums, and respiratory tract must be ruled out. The extreme sensitivity of both the benzidine and guaiac tests result in many positive reactions from minute amounts of blood present from extragastro-intestinal tract origin. These so-called false positive reactions have led to the devising of less sensitive techniques such as the Levin and Watt modification of the benzidine test which appears to be about 50 per cent less sensitive and therefore, when positive, likely to be more significant of a gastro-intestinal tract lesion. Also, the Alvarez and Wight modification of Gregersen's test which is sensitive to 1 part of blood in 200,000 is most useful.

The guaiac test is considerably less sensitive than the benzidine and for this reason many laboratories routinely apply both tests when an examination for occult blood is requested. The results of both tests are reported for such evaluation as the clinician may care to make.

Neither the benzidine nor the guaiac tests are specific for hemoglobin and therefore are not specific for blood. The presence of purulent material, iodides, and bromides gives false positive reactions. Thus, a negative reaction is of more absolute value than a positive reaction.

All cases of *ulcerated* carcinomas—stomach and colon—give continuously positive tests for occult blood. A non-cancerous gastric ulcer is more likely to give only intermittent positive test for occult blood. Also,

several of the intestinal parasitic infections cause intermittent or constant positive tests for occult blood in the feces.

Bile Pigments

As previously indicated, the brown color of feces is due to the formation of stercobilin, which is identical with urobilin found in urine from bilirubin. Urobilinogen is formed by reduction and is present in feces as well as in urine, but it is colorless. Both stercobilin and urobilinogen are present in the feces in increased amounts in conditions of accelerated red blood cell destruction, but technically the quantitative determination is more easily performed on urine. In patients who have had complete biliary obstruction the detection of small amounts of urobilin in the feces chemically is of value, for it signals the relief of a complete obstruction. Schmidt's mercuric chloride qualitative test is perhaps most widely used.

Trypsin

The absence or marked decrease of trypsin in the feces is a significant finding in the diagnosis of fibrocystic disease of the pancreas. Quite simple tests for the presence of trypsin in feces have been devised which are based on the ability of trypsin to liquify the gelatin layer of an X-ray film. These simple tests now are being increasingly employed as screening tests for the diagnosis of pancreatic deficiency. The administration of pancreatin will give a false positive. Trypsin is most active at a pH of 8, but most fecal emulsions with tap water are between 6 and 8, so ordinarily no adjustment of the pH need be made. When a negative test is obtained a more careful examination for the pancreatic ferments is indicated and these, including all quantitative studies, are best performed on duodenal contents. (See Chapter 23, Gastric and Duodenal Contents.)

MICROSCOPICAL EXAMINATION

Smears

Careful examination of appropriately prepared smears of fecal emulsion are perhaps the single most valuable type of fecal examination, for significant information may be obtained establishing specific parasitic infections or the presence of inflammatory lesions.

Cells

Excessive numbers of epithelial cells are present in inflammatory lesions. The number of pus cells also is increased markedly. The type

of cellular exudate in a dysenteric stool is of value in differentiating amebic dysentery from bacillary dysentery or ulcerative colitis. In uncomplicated amebic dysentery the exudate consists mainly of mucus and red blood cells, with a few polymorphonuclear and large mononuclear leukocytes. These latter cells may be degenerated, with only the nucleus remaining. The red blood cells are often in clumps. Charcot-Leyden crystals are often present. In bacillary dysentery and ulcerative colitis the exudate consists of blood and pus cells intimately mixed and often many large mononuclear cells. These may contain red blood cells. They often show degeneration of the nucleus with the cell membrane intact and may contain pink colored vacuoles.

The presence of eosinophils suggests an allergic condition, and of course clumps of malignant cells may be identified in cases of ulcerating neoplastic lesions of the lower intestinal tract. Red blood cells can be identified from bleeding lesions in the colon and rectum. The digestive processes destroy the cellular structure of red blood cells originating above the colon.

Crystals

Numerous crystals are normally present, depending upon the diet, such as triple phosphate and calcium oxalate, fatty acid, soaps, and neutral fat. Clear, colorless, slender, sharp, often needlelike, Charcot-Leyden crystals are frequently found associated with ulcerative intestinal lesions, particularly amebiasis. In cases of hemorrhage, the yellowish or brownish needle or rhombic form of hematoidin crystals can be identified.

Undigested Food

Normal feces contain undigested vegetable cells, fibers, and hairs, particularly from fruit. In diarrhea, particles of meat fibers and starch granules may be recognized. Mineral oil appears as highly refractory, round, or irregular globules.

EXAMINATIONS FOR EGGS AND LARVAE

Stools of normal consistency are most suitable but liquid diarrheal stools and specimens obtained after enema may be used, although eggs and larvae are less concentrated.

Smears

Several smears of fecal emulsion should be examined before calling a specimen negative. Thin smears are best in examinations for eggs, while

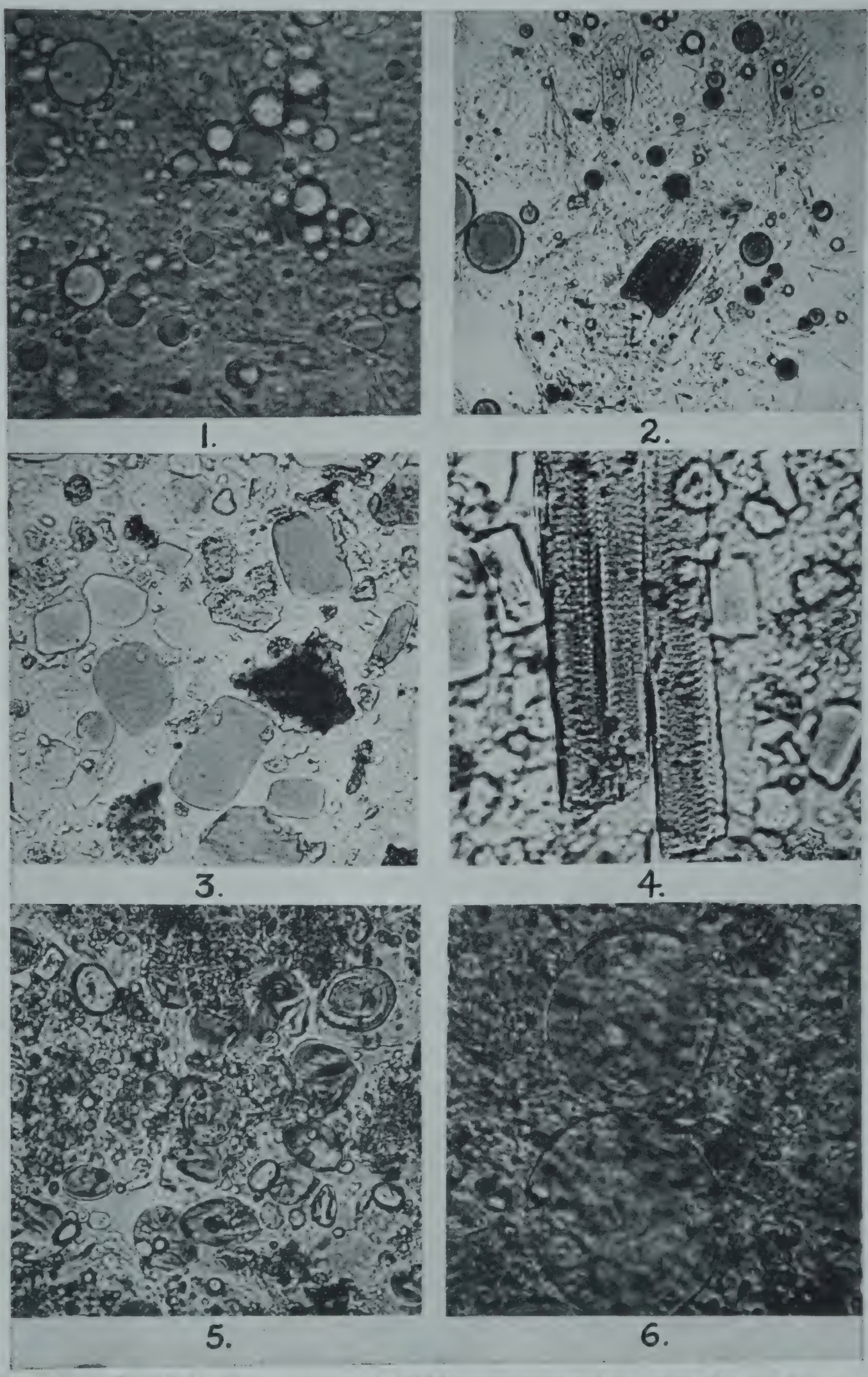


FIG. 25.1. Fecal food remnants (× 300). *1*, neutral fat droplets. *2*, neutral fat droplets and soap crystals. *3*, well digested meat remnants. *4*, nondigested meat remnants. *5*, starch granules under low power (16 mm.) magnification. *6*, starch granules under high power (4 mm.) magnification.

thicker ones are preferable in examinations for motile larvae such as Strongyloides.

All the intestinal worms lay eggs or deposit larvae in the intestinal canal except Trichinella, which deposits its larvae in the intestinal tissue. Most worms produce large numbers of eggs which are morphologically characteristic and readily identifiable. Recognition and identification of a particular egg establishes the presence of a specific parasitic worm infection in that patient.

Concentration of Eggs

When only a few adult female worms are present, eggs are sometimes too few to be found readily by direct smears. Therefore, after several negative smears, portions of the stool should be selected for concentration. Concentration techniques are of two general types. In one, the centrifugation, the eggs, being heavier than the medium they are suspended in (normal saline or tap water), are collected in the sediment after centrifuging and appropriate smears of this sediment are examined. The second concentration technique is by flotation. A portion of feces is emulsified in a concentrated salt solution of high specific gravity and the eggs, being lighter, rise to the top, float on the surface, are "skimmed off," and appropriate smears made for examination. The flotation techniques, while excellent for most eggs, regularly fail to demonstrate unfertilized Ascaris eggs, *S. japonicum* eggs, and some of the operculated eggs, while the centrifugation techniques are capable of demonstrating all intestinal helminth eggs present in feces.

Identification of Eggs

For a description of the identifying characteristics of eggs and larvae of the various intestinal helminths (worms), see Chapter 14, Medical Parasitology.

EXAMINATION FOR INTESTINAL PROTOZOA AND FLAGELLATES

Smears

Smears of fecal emulsions should be examined for the motile vegetative forms, trophozoites, and cysts. Techniques of culture, preservation, staining, and the identifying characteristics of these trophozoites and cysts are discussed in Chapter 14, Medical Parasitology. It is well to emphasize here the absolute necessity for special training of all individuals performing these examinations. For example, the differentiation of *E. histolytica* from the harmless widespread *E. coli* requires some

experience and training or many very sad mistakes are inevitable. Also, although no significant mycotic infections are diagnosed by fecal examination, yet yeast cells, spores of molds, and particularly *Blastocystis hominis*, must not be mistaken for a protozoan cyst.

EXAMINATION FOR PATHOGENIC BACTERIA

The techniques of isolation, identification, and significance of pathogenic bacteria in feces are discussed in Chapter 12, Medical Bacteriology.

INTERPRETATION

The average diagnostic laboratory, mostly because of offensive odors, has been little interested in fecal examinations. Positive findings, except for occult blood, are rare indeed, and obviously the great volume of negative reports is both valueless and too frequently misleading. For the physician to properly evaluate laboratory reports of fecal examinations, he must know whether the laboratory has a trained experienced worker using standard techniques or whether stool examinations are performed in a haphazard manner by an individual with no special training or aptitude. Since the eggs of intestinal parasites are easily identified, a positive report is highly significant. However, a negative report generally is of little or no value. Unless the worker has special training in the laboratory identification of intestinal protozoa, both positive and negative reports are valueless. This is particularly true in the diagnosis of amebiasis, where both trophozoites and cysts of the harmless *E. coli* are frequently misdiagnosed *E. histolytica* by the inexperienced worker.

In the chapters on Medical Bacteriology and Medical Parasitology the diagnostic merits and significance of these examinations are discussed in detail. It is appropriate here to emphasize again that repeated stool examinations are frequently necessary to establish the presence or absence of bacterial and parasitic infection.

A positive report for occult blood requires careful check to rule out the possibilities of an extra-intestinal origin of minute quantities of blood. Again, it is appropriate to emphasize the information that may be gained, regarding gastro-intestinal tract malfunction and disease, by a careful inspection of the feces. Definite leads on the efficiency of the digestive processes and evidence of significant alteration in motility, if present, are easily evident. The shape and form of the feces may show evidence of increased spasticity or rectal stricture The presence of gross blood and mucus is pathological and generally significant of colonic or rectal

pathology, and irritative and ulcerative lesions must be carefully sought for. The absence of bile pigments is easily established and signifies either obstruction or the failure of secretion of bile. In planning extensive diagnostic studies of the gastro-intestinal tract, always secure the necessary fecal studies first for the results will, in some cases, eliminate the necessity for the more expensive and arduous X-ray and liver function studies. Moreover, barium used in the X-ray studies persists in the feces for several days, changing the normal color and interfering with the microscopical examinations.

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